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Regulation of Protein Synthesis Factor EF-1α in *Mucor racemosus*†

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The protein synthesis elongation factor EF-1α of *Mucor racemosus* hyphae contained eight or nine methylated amino acids per molecule, whereas the factor from sporangiospores was nonmethylated. During the course of spore germination, the specific activity of the factor in crude extracts increased sixfold. This increase in activity was accompanied by a constant level of EF-1α-specific mRNA and a constant level of EF-1α protein. Methylation of the protein, however, accelerated during the germination process, in parallel with the increase in specific activity of the factor. We propose that the activity of EF-1α is regulated during germination through methylation of the protein and does not involve transcriptional regulation.

Mucor racemosus is a dimorphic fungus that grows as a budding yeast in CO_2 and as a mycelium in air. These alternative forms of growth, as well as the process of spore germination, are accompanied by differences in the translational system. Among these are differences in the rate of peptide bond formation (18, 19), the extent of phosphorylation of ribosomal protein S-6 (11, 12), and the methylation of the elongation factor EF-1α (9). In the latter case, we have found that of the 44 lysine residues, 8 or 9 are methylated when the organism grows in the mycelial form (9). The extent of methylation is less in the yeast form, and spores contain an EF-1α that is essentially nonmethylated.

In an effort to understand the regulation of EF-1α synthesis and activity and the role EF-1α may play in changes in the rate of peptide bond formation, we studied the acquisition of EF-1α activity during spore germination. These studies were aided by obtaining a cDNA clone which made it possible to measure mRNA levels. We show here that EF-1α protein and mRNA are made at constant levels during a period that EF-1α activity is rising dramatically. Our conclusion is that activity of EF-1α in *M. racemosus* is regulated through the methylation of the protein after synthesis.

MATERIALS AND METHODS

Organisms and culture conditions. *M. racemosus* (*M. lusitanicus*) ATCC 1216B was used in all experiments. Sporangiospores were prepared as described previously (20). Spores (2×10^5/ml) were germinated in YPG medium (2% glucose, 1% Bacto-Peptone, 0.3% yeast extract [pH 5.5]). The cultures were maintained at 28°C on a rotary shaker water bath and purged with at least 2 volumes of air per min.

EF-1α activity. EF-1α activity was assayed by the [^{3}H]Phe-tRNA binding assay and by stimulation of polyuridylate-directed polyphenylalanine synthesis. The EF-1α-mediated binding of [^{3}H]Phe-tRNA to ribosomes was assayed as described by Slobin and Moller (22), except that the assay buffer consisted of 20 mM Tris (pH 7.5), 25 mM KCl, 10 mM $MgSO_4$, and 2.5 mM mercaptoethanol. When guanylyl-imidodiphosphate was used in place of GTP, the reaction was stopped with 50 μl of 4% glutaraldehyde to stabilize the EF-1 complex bound to the ribosome. Polyuridylate-directed phenylalanine synthesis was conducted as described by Merrick (16), except that the buffer contained 50 mM KCl and 0.8 mM spermidine. [^{3}H]Phe-tRNA was prepared from yeast tRNA Phe (Boehringer Mannheim Biochemicals) and L-[2,6-^{3}H]Phe (Amersham Corp.) (50 Ci/mmol) with *Escherichia coli* crude aminoacyl-tRNA synthetases (16). High-salt washed ribosomes were prepared as described by Larsen and Sypherd (11), except that NH_4Cl was substituted for KCl. Elongation factor EF-1α was prepared as previously described (9), and EF-2 was prepared from wheat germ as described by Legocki (13). Cell extracts for the assay of EF-1α activity were prepared in 20 mM Tris (pH 7.5)–25% glycerol–0.1 m EDTA–5 mM mercaptoethanol. Protein was determined by the Bradford procedure (3).

EF-1α protein level. Germinating spores were labeled continuously with [^{35}S]methionine (25 μCi/ml of culture), and 1-ml samples were removed at various times. The cells were harvested by centrifugation, washed with water, and then broken with glass beads. The crude extract was fractionated by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell et al. (17). Proteins were visualized by Coomassie staining. The EF-1α spot was cut from the gel and hydrolyzed in 0.5 ml of 30% hydrogen peroxide, and the radioactivity was measured. To determine the total radioactivity in the gel, the gel was dehydrated in ethanol and digested in 10 ml of 30% hydrogen peroxide, and a sample was counted. The amount of EF-1α protein as a proportion of total cellular protein was determined as the ratio of radioactivity in the EF-1α spot to the total gel.

Quantitation of EF-1α mRNA. The levels of EF-1α mRNA were quantitated by in vitro translation and by slot blot hybridization. Bulk RNA was prepared by the procedure of Alton and Lodish (1). The RNA was translated with rabbit reticulocyte lysate (Bethesda Research Laboratories, Inc.), and the proteins were labeled with [^{35}S]methionine. The amount of EF-1α protein synthesized was quantitated by two-dimensional electrophoresis as described above.

For hybridization analysis, the RNA samples were bound to nitrocellulose as described by White and Bancroft (26), except that a slot blot manifold (Schleicher & Schuell, Inc.) was used. Hybridization conditions were those of Thomas (24). The EF-1α-specific cDNA probe (C. Katayama, T. Leathers, J. Linz, and P. S. Sypherd, manuscript in prepa-

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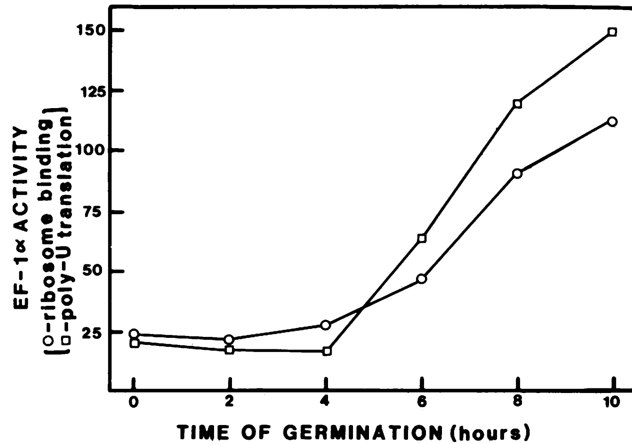

FIG. 1. Changes in EF-1α activity during germination. Spores were inoculated into germination medium at zero hour, and cell samples were harvested at the indicated times. The amount of EF-1α in these samples was determined by the ribosome-binding assay (22) (expressed as picomoles of EF-1α per milligram of protein) and by the stimulation of polyuridylate-directed translation (16) (expressed as picomoles of phenylalanine incorporated per minute per milligram of protein). Germ tubes emerged at 6 h, and germination was completed by 8 to 10 h.

ration) was labeled by nick translation (15). Autoradiographs were quantified by densitometry.

Methylation analysis. Germinating spores were pulse labeled for 1 h with [^{14}C]lysine, and the methyllysine content of EF-1α was determined as described previously.

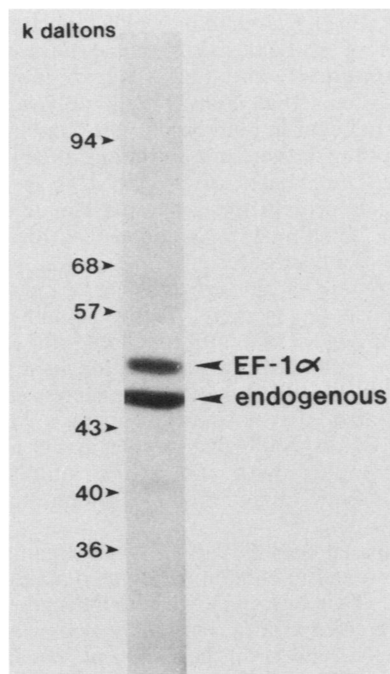

FIG. 2. Specificity of EF-1α cDNA probe. mRNA complementary to the cDNA probe was selected by hybridization and then translated in vitro. The translation products were mixed with purified EF-1α and subjected to polyacrylamide gel electrophoresis. The autoradiograph of the polyacrylamide gel is shown.

RESULTS

Acquisition of EF-1α activity during spore germination. The activity of EF-1α was determined in crude extracts during the course of spore germination by measuring both [^{3}H]Phe-tRNA binding to ribosomes and polyphenylalanine synthesis in the presence of polyuridylic acid. These data (Fig. 1) show that the specific activity of EF-1α increased about sixfold during the germination process. Protein accumulation during this same time proceeded at a relatively constant rate (18).

EF-1α mRNA levels during spore germination. The level of regulation, transcriptional or post-transcriptional, was determined in two ways. First, a cDNA probe for EF-1α was prepared. The cDNA, prepared in pBR322, was specific for EF-1α, as evidenced from the hybrid selection of mRNA. Figure 2 shows the results of in vitro translation of mRNA recovered from total polyadenylate RNA by hybridization to the cDNA. That only EF-1α and the endogenous rabbit reticulocyte protein were synthesized was confirmed by two-dimensional gel electrophoresis. The cDNA for EF-1α was used to measure the amount of EF-1α mRNA during spore germination. The mRNA levels changed very little during the time that EF-1α activity underwent its large increase (Fig. 3). The quantitation of slot blot hybridization data is shown in Figure 3. The amount of EF-1α mRNA was also determined by in vitro translation and quantitation of the amount of EF-1α synthesized. These results, expressed as the percentage of the total protein synthesized in vitro, are also given in Fig. 3. The results of specific hybridization and in vitro translation lead to the firm conclusion that EF-1α mRNA levels changed very little during spore germination and did not reflect the sixfold change in EF-1α activity.

Relative amount of EF-1α protein synthesized during spore germination. A constant, high level of EF-1α mRNA during the time that activity was changing raised the question of whether the EF-1α protein was being synthesized at similarly constant levels. This question was approached by germinating spores in [^{35}S]methionine and determining the relative amount of EF-1α protein from its unique position in a two-dimensional polyacrylamide gel (9). We found that the

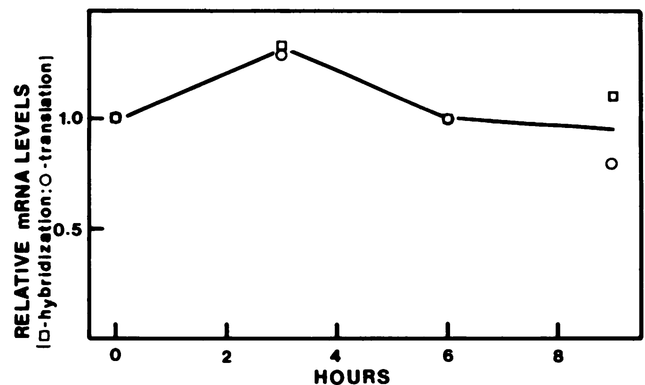

FIG. 3. Changes in EF-1α mRNA during germination. RNA was prepared from cells harvested at the indicated times after inoculation of the germination medium. The amount of EF-1α mRNA was quantitated by densitometric scanning of slot blots or by in vitro translation of the RNA. The message levels are expressed relative to the amount of mRNA present in the spores, which was designated as 1.0. EF-1α mRNA constituted 1.13 ± 0.20% of the translatable mRNA in spores.

amount of EF-1α protein, measured as a proportion of total cellular protein, changed little during spore germination (Table 1) and did not reflect the change in specific activity of the factor.

Extent of EF-1α methylation. A conventional conclusion from the foregoing data would be that EF-1α activity appears in germinating spores as a consequence of posttranslation modification. We tentatively ruled out a zymogen-enzyme type transition because the electrophoretic characteristics of the protein from spores were indistinguishable from those of germlings. From earlier work we knew that spore EF-1α was virtually nonmethylated (9). Accordingly, we studied the kinetics of EF-1α methylation during the germination period. These data (Fig. 4) show that the extent of methylation, measured as a proportion of the lysine residues present as mono-, di-, or trimethyllysine, increased in parallel with the rise in activity. It is therefore difficult to avoid the conclusion that methylation of either a certain number of lysines, or more likely certain critical lysine residues, is responsible for the activation of EF-1α. Our early attempt to resolve this point by isolating spore and germling EF-1α has not been entirely successful. Although the spore (nonmethylated) preparation consistently showed a three- to fourfold-lower specific activity than the germling (methylated) preparation, it was necessary to use different purification schemes from each source. This apparently results from the germling factor being part of a larger complex. Nevertheless, it is clear from the data presented here that although EF-1α activity in crude extracts varies as a function of morphogenesis, the levels of mRNA and EF-1α protein remain relatively constant.

DISCUSSION

The regulation of EF-1α activity in *M. racemosus* is most pronounced during spore germination, although there is some regulation during the yeast-to-hypha conversion (9). The data reported here make it clear that the regulation of EF-1α activity during spore germination is not due to transcriptional or translational regulatory mechanisms. However, our tentative conclusion that EF-1α activity may be regulated by the methylation events does require more proof. Our work along these lines will concentrate on isolating and studying the factor in its nonmethylated state (from spores) and after undergoing various levels of methylation.

We know of no example of the acquisition of activity by a protein that has undergone *N*-methylation. In one case it has been suggested that the *N*-methylation of cytochrome *c* plays a role in the binding of that molecule to the mitochondrial matrix (21). However, many proteins that are known to be *N*-methylated interact directly or indirectly with nucleic acids. It is possible that the addition of methyl groups to the ε-amino group of lysine would provide a subtle means of regulating such interactions. The *N*-methylated lysines that are found in the myosin of muscle and of *Physarum* species are located in the globular domain of the molecule containing the active sites for ATP hydrolysis and interaction with actin (8; M. Venkatesan, V. Nachmias, and I. R. McManus, Fed. Proc. **34**:671, 1975). Calmodulin, which regulates the activity of a number of proteins having nucleotides as substrate, contains trimethyllysine (25). The *N*-methylation of ribosomal proteins that interact with polynucleotides has been shown to occur in both procaryotes and eucaryotes (4, 5, 10, 23). Although there are mutations affecting the methylation of all three sites of protein L11 in *E. coli*, the methyl-deficient protein appears to participate in the formation of fully functional 50S ribosomes (6). A mutation affecting the methylation of protein L3 of *E. coli*, in which *N*-methylglutamine is not formed, is reported to result in a defect in ribosome assembly. This is shown by a slower assembly rate for the mutant compared with the wild type (14). However, once the ribosomes are formed, the particles are fully active (14). Since methylation increases the basicity of lysine residues, it is not hard to imagine that the *N*-methylation of EF-1α could play a role in the ability of the factor to complex with the acidic aminoacyl-tRNA, ribosomes, and mRNA and thereby facilitate the hydrolysis of GTP.

It is anticipated that the EF-1α of all organisms and cell types will be found to contain methylated amino acids. This assumption is based on our own unpublished findings with EF-1α from *Neurospora crassa*, *Saccharomyces cerevisiae*, and HeLa cells, reports on the EF-1α of *Artemia salina* (2) and 3T3 cells (7), and the fact that the *E. coli* analog of EF-1α, EF · Tu, also contains a methylated lysine. The apparent ubiquity of EF-1α methylation suggests that these methylated residues play an important role in peptide formation. A systematic study of the methylated and non-

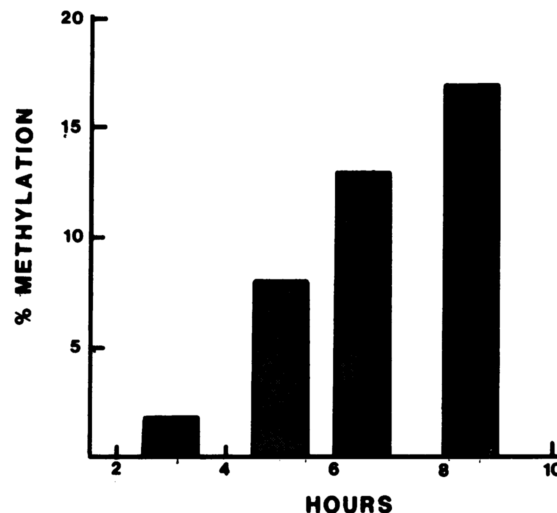

FIG. 4. Degree of EF-1α methylation. Germinating spores were pulse labeled with [^{14}C]lysine for the indicated time intervals. The labeled proteins were fractionated by two-dimensional gel electrophoresis, and the EF-1α spot from the gel was analyzed for methyllysine. Values represent the percentage of lysine residues methylated.

TABLE 1. Relative level of EF-1α protein in germinating spores

Time of germination (h)	EF-1α[a] (% of cell protein)
0[b]	1.07 ± 0.13
2	2.79 ± 0.26
4	1.97 ± 0.25
6	3.30 ± 0.26
8	2.53 ± 0.21
10	2.13 ± 0.21

[a] Germinating spores were labeled with [^{35}S]methionine, and the labeled proteins were fractionated by two-dimensional electrophoresis as described in the text. Values represent the percentage of counts per minute in the EF-1α spot of the gel ± the standard error.

[b] Zero hour value was obtained from sporangiospores formed from hyphae growing on [^{35}S]methionine-containing medium.

methylated forms of EF-1α from *M. racemosus* should provide insight into this role.

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