Eukaryotic Initiation Factor 4D, the Hypusine-containing Protein, Is Conserved among Eukaryotes*

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The uncommon amino acid hypusine (N\(^{\text{ε}}\)-[4-amino-2-hydroxybutyl]lysine) was discovered nearly 20 years ago in acid extracts of bovine brain (1), but has only recently been found as part of a particular protein. When lymphocytes were grown in medium containing \([^{3}H]\)spermidine, a single radioactive protein with a molecular weight of approximately 18,000 was detected. The radioactivity in this protein was shown to be part of a particular protein. When lymphocytes were grown in medium containing \([^{3}H]\)spermidine, a single radioactive protein with a molecular weight of approximately 18,000 was detected. The radioactivity in this protein was shown to be part of a particular protein.

When mammalian cells are grown in medium containing \([^{3}H]\)spermidine, a single major tritiated protein identical to eukaryotic initiation factor 4D becomes labeled. This protein contains 1 residue/molecule of tritiated \((N^{\text{ε}})-(4\text{-amino-2-hydroxybutyl})\)lysine, a rare amino acid which has been found in no other protein. In order to investigate the conservation of this protein, we examined two nonmammalian eukaryotes, the yeast *Saccharomyces cerevisiae* and the insect *Drosophila melanogaster*, and the eubacterial prokaryote *Escherichia coli* for the presence of the hypusine-containing protein. When the eukaryotic cells were grown in the presence of \([^{3}H]\)spermidine, electrophoretic analysis revealed a single labeled protein. In each case, the apparent molecular weight was near 18,000 and the relative pl was approximately 5.2, similar to the hypusine-containing protein of mammals. Amino acid analysis confirmed the presence of tritiated hypusine in each case, and silver staining of two-dimensional polyacrylamide gels demonstrated that, in yeast and fruit flies as in mammals, the protein is relatively abundant. In the eubacterium *E. coli*, one tritiated protein was predominant, but its molecular weight was 24,000 and we found no evidence that it contained tritiated hypusine. We found no evidence for the existence of the hypusine-containing protein in the archaebacterium *Methanococcus voltae*. These data suggest that the hypusine-containing protein is conserved among eukaryotes.

The uncomon amino acid hypusine (N\(^{\text{ε}}\)-[4-amino-2-hydroxybutyl]lysine) was discovered nearly 20 years ago in acid extracts of bovine brain (1), but has only recently been found as part of a particular protein. When lymphocytes were grown in medium containing \([^{3}H]\)spermidine, a single radioactive protein with a molecular weight of approximately 18,000 was detected, and the radioactivity in this protein was shown to be due entirely to a single hypusine residue (2). Further studies elucidated a number of details of the post-translational synthesis of hypusine from protein-bound lysine, with spermidine the immediate amine precursor and deoxyhypusine an intermediate (3-5).

Interest in the hypusine-containing protein was stimulated by its identification as eukaryotic initiation factor 4D (eIF-4D) (6), a protein found in the salt wash of rabbit reticulocyte ribosomes, whose function is uncertain (for review, see Refs. 7 and 8). This protein has been found in all tissues and cells (9) of every mammalian investigated (9-16). We have investigated the extent of conservation of this protein in bacteria, fungi, and insects. In this report, we present the first evidence for the presence of the hypusine-containing protein in two evolutionarily distant nonmammalian eukaryotes, the fruit fly *Drosophila melanogaster* and the unicellular fungus *Saccharomyces cerevisiae*. Our results suggest conservation of this protein among eukaryotes.

**EXPERIMENTAL PROCEDURES**

Materials—Sequana grade pyridine, triethylamine, and phenylisothiocyanate were from Pierce Chemical Co. HPLC-grade acetonitrile was purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI); hydrochloric acid was Baker Analyzed reagent grade. Synthetic hypusine (16) was a generous gift of Dr. Tetsuo Shiba, Osaka University, Osaka, Japan. Difluoromethylornithine was kindly provided by Dr. Peter McCann (Merrell Dow). \([^{3}H]\)Spermidine \((\text{terminal methylene}-^{3}H(N))\), \(15 \muCi/\mumol\), was obtained from Du Pont-New England Nuclear.

**Drosophila and yeast** tissue culture cells of Schneider’s line 2 were maintained in Shield’s and Sang’s medium as described previously (17). HeLa cells were maintained in monolayer culture and grown under standard conditions in Eagle’s minimum essential medium (GIBCO) containing 10% fetal calf serum. Experiments with *S. cerevisiae* were performed with strain A31 (13) or LM-1 (18). Experiments with *Escherichia coli* employed strain J1503 (19).

**Protein Labeling**—For all eukaryotic species, preincubation with the irreversible inhibitor of ornithine decarboxylase, difluoromethylornithine was employed to deplete endogenous, intracellular polyamine pools (22) and to increase the specific activity of protein labeling. For all species, cells were harvested from late logarithmic phase growth.

For labeling, *Drosophila* cells were seeded into complete Shield’s and Sang’s medium on day 1. On day 2, they were transferred to complete medium containing 5 mM difluoromethylornithine. On day 3, they were transferred to medium minus yeastolate and serum and containing 20-100 mCi/ml \([^{3}H]\)spermidine, in which they remained until they were harvested on day 4.

HeLa cells were seeded as monolayers in standard medium containing 10% fetal calf serum on day 1. On day 2, they were transferred to complete medium containing 5 mM difluoromethylornithine. On day 3, they were transferred to medium minus yeastolate and serum and containing 20-100 mCi/ml \([^{3}H]\)spermidine was added, and the cells were harvested from this medium on day 4.

1. The abbreviations used are: eIF-4D, eukaryotic initiation factor 4D; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PTC, phenylthiocarbamid.

2. Spermidine trihydrochloride, \((\text{terminal methylene}-^{3}H(N))\) H\(_2\)NCH\(_2\)CH\(_2\)NHCH\(_2\)(CH\(_3\))\(_2\)CH\(_2\)NH\(_2\)·3HCl.

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Starter cultures of yeasts were grown overnight in rich, acetate-containing medium (1% Bacto yeast extract (Difco), 2% Bactopeptone (Difco), and 1% potassium acetate). On day 2 they were transferred to synthetic acetate medium (1% potassium acetate, 0.67% Bacto yeast nitrogen base (Difco), 5% phthalic acid, pH 5.5, supplemented with the appropriate auxotrophic requirement) which was made 5 mM in difluoroornithine. On day 3 cells were pelleted and transferred to synthetic acetate medium containing 20–100 mM C18(5) spermidine in which they remained 12–18 h until they were harvested.

Starter cultures of E. coli were grown overnight in minimal A medium (20). Cells were collected by centrifugation, and transferred into fresh medium to which 20–100 mM C18(5) spermidine had been added. They remained in labeling medium for 4–8 h until harvesting. Starter cultures of Methanothermobacter voltae were grown overnight in a defined medium (21), under an atmosphere of H2 and CO2 (80:20), to an optical density of 0.3–0.7 at 660 nm, as measured in a Spectronic 21 (Bausch and Lomb). They were then diluted 1:10 to into 5 ml of fresh medium to which 100 mM C18(5) spermidine had been added and grown with shaking at 30 °C to a density of ~0.5 until harvesting by centrifugation.

CNBr Cleavage—Ethanol-precipitated proteins from all species were dried under nitrogen and dissolved in 70% formic acid, with or without 0.5% (w/v) CNBr. (Ten-fold higher concentration of CNBr yielded identical cleavage patterns.) These solutions were incubated at room temperature overnight, then dried under nitrogen. The peptides so produced were then processed for separation by one-dimensional SDS-PAGE.

Protein Extraction and Electrophoresis—From yeasts, M. voltae, and E. coli cells, proteins were extracted by lysis with glass beads in 95% ethanol containing 1 mM phenylmethylsulfonyl fluoride, as described previously (23). Proteins from both HeLa and Drosophila tissue culture cells were precipitated with cold 10% trichloroacetic acid, then washed with ice-cold 95% ethanol (24, 25).

For one-dimensional analysis, proteins were separated by 15% SDS-PAGE (26). Two-dimensional analysis was performed according to the method of Lee et al. (27) and Andersen and Anderson (28, 29), employing mixed ampholytes (0.8% of Sevalyte, pH 3–10; 0.8% of Pharmalyte, pH 3–10; and 0.4% of LKB Ampholine, pH 5–7). The second dimension separation was performed on a linear 9–18% polyacrylamide gradient gel. Gels were prepared for fluorography according to the method of Laskey and Mills (30). Silver staining was performed according to manufacturer’s instructions (Bio-Rad).

### Amino Acid Analysis

The region of the Coomassie Blue-stained gel at the position of the appropriate molecular weight marker was excised, dried, and subjected to hydrolysis in 6 N HCl at 110 °C for 24 h. The volume of acid used was sufficient to hydrate the entire gel slice. Following hydrolysis, the sample was chilled at −10 °C for 6–12 h, and all insoluble material was pelleted by centrifugation at 5000 × g. The supernatant was evaporated to dryness in a Speed Vac concentrator and dissolved in 100 μl of acetonitrile:pyridine:triethylamine:H2O (10:5:2:3). This solution was dried to remove the remaining HCl and the residue was redisolved in 100 μl of coupling buffer. To this solution was added 5 μl of phenylisothiocyanate and the reaction was allowed to proceed at room temperature for 15 min. To volatilize unreacted reagents and side products, the samples were evaporated to dryness and re-evaporated twice more after being redisolved in 200 μl of acetonitrile. The resulting PTC-amines and -amino acids were dissolved in 70% (v/v) acetonitrile-water and any insoluble material was pelleted by centrifugation at 17,000 × g prior to reverse-phase liquid chromatography of the supernatant. In all cases, the radioactive material was completely soluble in the injection solvent mixture. The spectrophotometric yields and relative retention times of the polyamine or amino acid derivatives examined were consistent with complete phenylisothiocyanate derivatization of all primary and secondary amines in all molecular species.

Separation and identification of PTC derivatives was performed using an IBM LC/9533 ternary gradient system, a Perkin-Elmer LC-85 detector, and a Nelson Analytical Series 4400 data system. The temperature of the column was maintained at 52 °C, and flow rate was 2 ml/min. For identification of radioactive PTC-amines and -amino acids, fractions were collected directly into scintillation vials using a Pharmacia Frac-100 fraction collector. Reverse-phase chromatography was performed using an IBM octadecylsilane column (4.6 × 250 mm). The eluent gradient is described in Table I. These conditions achieved base-line separation of the following PTC derivatives (in order of elution times): di-PTC-ornithine, di-PTC-putrescine, tri-PTC-hypusine, tri-PTC-spermidine, and tetra-PTC-spermine. In most experiments, unlabeled PTC-hypusine (31) was co-injected with the radiolabeled sample and served as an internal standard. Recovery of radioactivity from the reverse-phase column was >95%.

### RESULTS

In order to investigate the conservation of the hypusine-containing protein, we labeled S. cerevisiae, D. melanogaster tissue culture cells, and HeLa cells with [3H]sperrmidine (terminal methylene-3H(N)). HeLa cells were used to provide a standard, since the hypusine-containing protein of mammals has been previously characterized. From each species, proteins were extracted, separated by SDS-PAGE, and visualized by fluorography. Fig. 1 shows that only one protein from each species was labeled. The labeled proteins from the human HeLa cell line, the D. melanogaster tissue culture cells, and the yeast S. cerevisiae all had apparent molecular weights of approximately 18,000, although the HeLa protein consistently migrated slightly further than that of the other two species. These results provided initial evidence that the hypusine-containing protein was present in these lower eukaryotes.

We next performed two-dimensional electrophoretic analysis on total cellular proteins from these three species. Each type of cell was incubated in the presence of [3H]spermidine as described above, proteins were electrophoretically separated, and duplicate gels were processed either for fluorography or for silver staining. The results are shown in Fig. 2. For all species, fluorography showed similar results, with the tritiated proteins having apparent molecular weights near 18,000. Furthermore, the proteins from all three species had similar acidic PI values: 5.1–5.2 for the yeast protein and 5.3–5.5 for the Drosophila and HeLa proteins. We also observed that in each species the hypusine-containing protein was relatively abundant, since this protein was readily seen as a prominent silver-stained spot.

Isoelectric focusing separated two forms of the tritiated protein, which are visible in both the fluorograms and the silver-stained proteins of from all three eukaryotic species studied. (Such a result confirms previous observations with the mammalian protein (6, 10).) In each case, the more intense spot was the more basic of the pair, and we estimate that it is 10 times as intense as the more acidic spot. Carbamylation might produce such extra spots artifically, but examination of the silver-stained gels revealed no evidence of carbamylation of any other protein. Furthermore, samples of silver-stained gels of proteins from various tissues of human and rat always revealed similar paired spots. Whether the protein does in fact exist as two isoforms in vivo or whether it is an artifact of experimental method, the consistent presence of

![Graph](image-url)
the two spots in identical ratios in samples from mammals, yeast, and fruit flies provides additional evidence for conservation of the hypusine-containing protein among these organisms.

We next asked whether the tritium label in these proteins was present in tritiated hypusine, since this unusual amino acid is a hallmark of the previously characterized mammalian eIF-4D. The labeled protein of each species was sliced from a wet, SDS-polyacrylamide gel, and the slices were dried and hydrolyzed by 6 M HCl. The hydrolysate was allowed to react with phenylisothiocyanate to derivatize amino groups and was subjected to amino acid analysis by reverse-phase chromatography. Parallel analyses were performed on ornithine, putrescine, spermidine, spermine, and authentic, chemically synthesized hypusine (30).

Fig. 3 shows a typical chromatogram. From all three eukaryotes, we found the same major peak of radioactivity produced which coelutes with standard, unlabeled hypusine. We conclude that the radioactivity in the 18,000-dalton proteins of yeast and Drosophila is due to tritiated hypusine for the following reasons. First, the radioactivity originated from [3H]spermidine and became covalently associated with an 18-kDa protein in an acid-stable fashion. (The acid stability shows that this radioactivity is not due to γ-glutamylspermidine, a molecule which cochromatographs with hypusine under certain conditions (2).) Second, the chromatographic distribution of radioactivity was the same for the lower eukaryotes as for HeLa, and it has previously been shown that the label of the mammalian protein is due to tritiated hypusine (6). Finally, the tritiated amino acid coeluted with the hypusine standard, even when chromatography was performed at pH 4.5 and 6.0, confirming that the peaks of radioactivity are due to radioactive hypusine.

The conservation of the labeled proteins was further investigated by subjecting them to CNBr cleavage. We grew cells in media containing [3H]spermidine, extracted total cellular proteins, and incubated them overnight in 70% formic acid with or without CNBr. The peptide fragments so produced were separated by SDS-PAGE and visualized by fluorography. Fig. 4 presents the fluorograms from labeled HeLa and yeast proteins, which show that the cleavage products are remarkably similar. It seems likely that the presence of multiple labeled peptides indicates incomplete cleavage (32). Because
Conservation of eIF-4D among Eukaryotes

Mr (X 10^-3)  Human  Yeast
45  -  +
24  -
18  -
14  -

FIG. 4. CNBr cleavage products of tritiated, hypusine-containing protein of human and yeast cells. Human (HeLa) and yeast cells were grown in the presence of [3H]spermidine and total cellular proteins were extracted, precipitated, and dried. They were then dissolved in 70% (v/v) formic acid with (+) or without (-) the addition of CNBr. After incubation at room temperature overnight, peptide fragments were dried under nitrogen, and then dissolved in SDS sample buffer and subjected to one-dimensional SDS-PAGE. 3H-Peptides were visualized by fluorography. The positions of migration of molecular size markers are shown on the left.

these data imply conservation of the relative positions of hypusyl and methionyl residues, they provide further evidence of the structural homology of the labeled yeast protein with the mammalian hypusine-containing protein. The cleavage pattern of the Drosophila protein did not resemble that of HeLa and yeast (not shown). Note that higher molecular weight bands were reproducibly observed, but only in formic acid-treated yeast samples.

We next looked for the presence of the hypusine-containing protein in prokaryotes. We repeated some of the experiments described above with the eubacterium E. coli, beginning with an examination of the radioactive proteins synthesized when cells of this species were grown in medium containing [3H]spermidine. The results of one-dimensional electrophoretic analysis are shown in Fig. 5. Just as in yeast, insect, and mammalian cells, only one small radioactive protein was produced, but its apparent molecular weight was 24,000, not 18,000. (Other minor bands were sometimes observed, but these were not reproducible.)

To investigate this protein further, we examined it for the presence of radioactive hypusine, as described above. The chromatogram which resulted showed no peak comigrating with authentic hypusine, although other unidentified peaks were produced. We also performed CNBr cleavage of the radioactive E. coli protein and obtained a fluorogram resembling none of the patterns we had obtained from eukaryotes (unshown).

We also looked for the presence of the hypusine-containing protein in archaeabacteria. We grew cells of M. voltae in defined medium (21) to which had been added [3H]spermidine. Electrophoresis of cellular proteins, followed by fluorography, showed that no strongly labeled proteins were produced, although a low background level of tritiated proteins suggested that uptake and metabolism of [3H]spermidine had occurred (unshown). Obviously, we cannot rule out the existence of the hypusine-containing protein in this kingdom without an examination of other taxa.

FIG. 5. One-dimensional electrophoretic analysis of E. coli proteins labeled with [3H]spermidine. E. coli cells were grown in the presence of [3H]spermidine. Total cellular proteins were extracted, separated by electrophoresis in the presence of SDS on 15% polyacrylamide slab gels, and visualized by fluorography. The distances of migration of molecular size markers are shown on the left.

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DISCUSSION

In this report, we have provided evidence that eIF-4D, the hypusine-containing protein, is conserved among eukaryotes. We have shown that with cells of the budding yeast S. cerevisiae and with the fruit fly D. melanogaster, as well as with the mammalian HeLa cell, incubation in medium containing [3H]spermidine [terminal methylene-3H(N)] results in the labeling of a single protein or pair of proteins of molecular weight approximately 18,000 and of relative pI near 5.2. We confirmed that the hypusine-containing protein of mammals is relatively plentiful (33, 34), for we found it to be a prominent spot by silver staining, and found that this protein is also abundant in the lower eukaryotes Saccharomyces and Drosophila. In addition, our amino acid analyses have demonstrated the presence of tritiated hypusine in each of these tritiated proteins. Finally, we have shown that the fluorogram of labeled peptides resulting from CNBr cleavage of the radioactive protein of yeast is indistinguishable from that of mammals, implying conservation of relative positions of hypusyl and methionyl residues. These distinct lines of evidence suggest that proteins closely related to the mam-
malian hypusine-containing protein may be present in all nucleated cells.

Our experiments that asked whether the hypusine-containing protein is present in *E. coli* yielded results with an uncertain interpretation. As with eukaryotes, one-dimensional electrophoretic analysis revealed a single labeled protein produced by *E. coli* when grown in the presence of labeled spermidine, but the apparent molecular weight of this protein was 24,000, while that of the eukaryotic proteins was approximately 18,000. Furthermore, amino acid analysis failed to provide evidence that this protein contained hypusine, and CNBr cleavage yielded a pattern of fragments that did not resemble that of any eukaryotic protein examined.

It is possible that the tritiated 24-kDa protein which we found in *E. coli* is not related to the hypusine-containing protein of eukaryotes. However, our data equally permit another interpretation, that the *E. coli* protein is a slightly larger relative of the protein of nucleated cells. Furthermore, while the *E. coli* protein apparently does not contain hypusine, it does contain some unusual amino acid derived in part from spermidine, perhaps the eukaryotic biosynthetic precursor, deoxyhypusine; we have not yet tested this hypothesis. In short, definitive interpretation of the results of our investigations of *E. coli* must await further data, such as comparative amino acid or nucleic acid sequences, or evidence of the existence of deoxyhypusine in the *E. coli* protein.

The conservation of the hypusine-containing protein among eukaryotes separated by 900 million years of evolution is remarkable, especially since such conservation implies not only retention of the structural gene but also the genes involved in the enzymatic synthesis of hypusine from lysine. Presumably, at least two enzyme activities, a butylaminotransferase and a deoxyhypusine hydroxylase, are involved in the unique post-translational modification of the hypusine-containing protein. That this conservation is so extensive makes the function of this protein an especially intriguing question. Our recent experiments, reported in the accompanying paper (35), have failed to confirm earlier data suggesting that changes in the presence or absence of hypusine in this protein might correlate with changes in protein synthesis (36). Early studies of the role of this protein in translation initiation have also failed to clarify its cellular role. Nevertheless, the results presented here suggest that this protein provides a function common to all eukaryotic cells. The powerful genetics methods available for studies in both *Drosophila* and *Saccharomyces* might prove useful in elucidating this function.

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