Characterization of Elongation Factor-1A (eEF1A-1) and eEF1A-2/S1 Protein Expression in Normal and wasted Mice*

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The eEF1A-2 gene (S1) encodes a tissue-specific isoform of peptide elongation factor-1A (eEF1A-1); its mRNA is expressed only in brain, heart, and skeletal muscle, tissues dominated by terminally differentiated, long-lived cells. Homozygous mutant mice exhibit muscle wasting and neurodegeneration, resulting in death around postnatal day 28. eEF1A-2/S1 protein shares 92% identity with eEF1A-1; because specific antibodies for each were not available previously, it was difficult to study the developmental expression patterns of these two peptide elongation factors 1A in wasted and wild-type mice. We generated a peptide-derived antiserum that recognizes the eEF1A-2/S1 isoform and does not cross-react with eEF1A-1. We characterized the expression profiles of eEF1A-1 and eEF1A-2/S1 during development in wild-type (+/+) heterozygous (+/wst), and homozygous (wst/wst) mice. In wild-type and heterozygous animals, eEF1A-2/S1 protein is present only in brain, heart, and muscle; the onset of its expression coincides with a concomitant decrease in the eEF1A-1 protein level. In wasted mutant tissues, even though eEF1A-2/S1 is absent, the scheduled decline of eEF1A-1 occurs nonetheless during postnatal development, as it does in wild-type counterparts. In the brain of adult wild-type mice, the eEF1A-2/S1 isoform is localized in neurons, whereas eEF1A-1 is found in non-neuronal cells. In neurons prior to postnatal day 7, eEF1A-1 is the major isoform, but it is later replaced by eEF1A-2/S1, which by postnatal day 14 is the only isoform present. The postdevelopmental appearance of eEF1A-2/S1 protein and the decline in eEF1A-1 expression in brain, heart, and muscle suggest that eEF1A-2/S1 is the adult form of peptide elongation factor, whereas its sister is the embryonic isoform, in these tissues. The absence of eEF1A-2/S1, as well as the on-schedule development-dependent disappearance of its sister gene, eEF1A-1, in wst/wst mice may result in loss of protein synthesis ability, which may account for the numerous defects and ultimate fatality seen in these mice.

Peptide elongation factor-1A (eEF1A-1)1 is an abundant protein, once thought to be ubiquitously expressed; its major role is to mediate the transfer of charged aminoacyl-tRNA to the A site of the ribosome during peptide elongation. We have previously shown that the mRNA expression of this gene declines in rat brain, heart, and skeletal muscle during development (1, 2), to the extent that in adult skeletal muscle, there is an almost complete loss of eEF1A-1 mRNA expression; a sister gene, eEF1A-2/S1, is expressed instead (3). Furthermore, in contrast to the ubiquitous expression of eEF1A-1 in many cell types, eEF1A-2/S1 expression is limited to the terminally differentiated cells of the brain, heart, and skeletal muscle (3, 4).

Recent evidence suggests that eEF1A-2/S1 is an alternative peptide elongation factor-1A for neurons and myocytes, because the amino acid homology between them is 92% (5, 6), and eEF1A-2/S1 can perform peptide elongation in vitro (7). The absence of eEF1A-2/S1 mRNA, as a result of a deletion in the promoter and first exon of the eEF1A-2/S1 gene, was recently identified in the mutant mouse wasted (8). wasted mice exhibit severe muscle wasting and degeneration of motor neurons, beginning at 3 weeks after birth and die by the age of 28 days (8–11). This coincides with the decline of eEF1A-1 expression in brain, heart, and skeletal muscle, without the normal concomitant appearance of its sister gene; because neither form of elongation factor-1A is found, the consequential loss of protein synthesis ability is the likely cause of death.

Distinguishing between eEF1A-1 and eEF1A-2/S1 protein expression was long hampered by the absence of isoform-specific antibodies; for example, it was impossible to determine the proportion of eEF1A-1 versus eEF1A-2/S1 in any given skeletal muscle biopsy. This is specifically true with regard to a recent report, which concludes that there is no change in eEF1A in young and old muscle (12); this conclusion was based on a study using a pan-monoclonal antibody that recognizes both isoforms and thus cannot discriminate either qualitatively or quantitatively between the two sister forms of this gene family. To investigate the presence of eEF1A-2/S1 protein and differentiate its level from that of its sister, during mouse development, we generated a polyclonal antiserum that specifically recognizes eEF1A-2/S1 isoform and does not cross-react with eEF1A-1. Using it along with another antiserum that specifically recognizes eEF1A-1, we characterized the protein presence of both peptide elongation factor-1A isoforms during development in normal and wasted mutant mice.

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1 The abbreviations used are: eEF1A, peptide elongation factor-1A; GST, glutathione S-transferase; PCR, polymerase chain reaction; TBS, Tris-buffered saline, 0.5% Tween 20; bp, base pair(s); kb, kilobase(s).
MATERIALS AND METHODS
Peptide Synthesis and Production of eEF1A-1- and eEF1A-2/S1-specific Antibodies—A highly purified, sequence-specific peptide of eEF1A-2/S1 protein, CB5 (see Fig. 1A), was obtained by high performance liquid chromatography on a C18 reverse-phase column, using an acetonitrile and trifluoroacetic acid gradient. CB5 protein was solubilized in 20 mM phosphate buffer and coupled to keyhole limpet hemocyanin carrier protein (Pierce) through amino-terminal cysteine residues. Antibodies were generated by multiple injections of 200 μg of the peptide in Freund’s incomplete adjuvant (Pierce), followed by two booster injections given at 3-week intervals with the same amount of antigen. One week after the final injection, the rabbits were sacrificed, and blood was collected. The eEF1A-1-specific antibody was raised against eEF1A-1 protein-specific peptide HT7 (see Fig. 1B), as previously described (13).

Tissue Preparation—Brain, heart, muscle, liver, and spleen samples were obtained from mouse, rat, and human autopsy tissues. Tissue extraction and preparation were performed as previously described (14), with minor modifications. All tissues were extracted by homogenization in a 2-ml tissue grinder (Wheaton, Millville, NJ) with 1 ml of homogenizing buffer (300 mM sucrose, 150 mM KCl, 30 mM Tris-HCl, 5 mM MgCl₂, 1.5 mM diithiothreitol, 1 mM EDTA, pH 8.0, and 1.5% Triton X-100) containing protease inhibitors (10 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride) (ICN Biochemicals Canada, Montréal, Quebec). Protein extracts were then incubated with 150 μg/ml DNase I (Roche Molecular Biochemicals) on ice for 1 h and sonicated for 1 min in an ice bath, followed by centrifugation at 3000 rpm for 15 min. The resulting pellet was discarded, and the supernatant was designated as total protein. Protein concentration was assayed by a Bio-Rad protein assay reagent (Bio-Rad), using bovine plasma globulin as a standard.

Expression and Purification of Recombinant Proteins—The construction of recombinant plasmids and expression of eEF1A-2/S1 protein in the glutathione S-transferase (GST) system using the pGEX-2T plasmid vector (Amersham Pharmacia Biotech) were reported previously, and the recombinant plasmid was referred to as GST-eEF1A-2/S1 (13). We also amplified the mouse eEF1A-1 cDNA by polymerase chain reaction (PCR) and subcloned it into the pGEX-2T expression vector, resulting in the GST-eEF1A-1 construct. The recombinant vectors were used to transform Escherichia coli. Individual clones were isolated, and the constructs were analyzed by DNA sequencing to identify those carrying the desired fragment, without any mutations that might have
been introduced by PCR. Extraction and purification of GST and GST fusion proteins (GST-eEF1A-2/S1 and GST-eEF1A-1) using glutathione-Sepharose 4B beads were performed as previously described (15).

**Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis** was carried out on 10 or 12% gels using a discontinuous Laemmli system (16). For each assay of protein profile determination, 50 μg of total protein extract was loaded onto the SDS-polyacrylamide gel. After electrophoresis, the proteins were either stained with Coomassie Brilliant Blue R-250 or transferred onto nitrocellulose paper. The nitrocellulose membranes were stained with Ponceau S (Sigma) to visualize the electrophoretic pattern of total protein and subsequently destained with Tris-buffered saline (50 mM Tris-HCl, 500 mM NaCl) containing 0.5% Tween 20 (TBST), followed by blocking with 5% milk in TBST for 1–2 h. The membranes were probed with the anti-eEF1A-1 or anti-eEF1A-2/S1 polyclonal antibodies for 2 h at room temperature in TBST at a 1:2000 dilution. The membranes were then washed four times with TBST for 10 min each, incubated with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Cappel, Durham, NC) for 1 h at room temperature in a 1:20,000 dilution, and washed four times as described above. Nitrocellulose blots incubated with horseradish peroxidase-conjugated secondary antibodies were developed using enhanced chemiluminescence detection (ECL) (Amer sham Pharmacia Biotech) according to the manufacturer’s protocols and exposed to x-ray film.

**Immunoprecipitation—** eEF1A-1 or eEF1A-2/S1 was immunoprecipitated from total protein extracts of adult rat brain, liver, and skeletal muscle, using a monoclonal anti-eEF1A antibody (17) (Upstate Biotechnology, Lake Placid, NY) that recognizes both proteins. One mg of total protein extracts was pre-cleared with 50 μl of protein G-agarose (Sigma) for 30 min at 4 °C. Of the pre-cleared tissue extract, 720 μg was incubated with 5 μg of anti-eEF1A monoclonal antibody for 17 h at 4 °C. Immune complexes were collected with 100 μl of protein G-agarose (Sigma) for 3 h at 4 °C. The protein G-agarose/immune complex was washed four times with 1 ml of ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, resuspended in 100 μl of SDS-loading buffer, and heated at 95 °C for 10 min. Twenty-five μl of the immunoprecipitated mixture was used for Western blotting analysis using the monoclonal anti-eEF1A-1, polyclonal anti-eEF1A-1, or anti-eEF1A-2/S1 antibodies.

**wasted Mice Genotyping—** A pair of heterozygous mice obtained from the Jackson Laboratory was further cross-bred. When the F1 offspring reached the age of day 7, ~0.5 cm of the tails of the pups was cut, and genomic DNA was isolated as described (18). Essentially, tails were incubated in 0.5 ml of lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg of proteinase K) overnight at 55 °C. DNA was extracted using phenol/chloroform, precipitated using isopropanol, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). One hundred μg of genomic DNA was amplified in 50 μl of reaction buffer containing 10 mM dNTPs, 100 ng of primers (designated P1), and 4 units of Taq DNA polymerase (PerkinElmer Life Sciences) using long distance polymerase chain reaction. Primers selected for identification of eEF1A-2/S1 gene presence were designed as follows: Primers 1 (P1), sense (5'-GACAGAGAAAGAGTAATGGC-3'); 4–24 base pairs (bp)) and antisense (5'-GGCCATCTTTGATTTGTTG-3'), 18814–18897 bp); primers 2 (P2), sense (5'-TAGGGCTTCGTTGGAACG-3'); 15752–15770 bp) and antisense (5'-CTACTCTCCCGAATGCTC-3'), 16204–16187 bp). Long distance PCR reactions were carried out at 95 °C for 3 min, followed by 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 3 min, for a total of 28 cycles. Regular PCR reactions in buffer containing 200 μg of genomic DNA, 100 ng of primers (P2), 10 mM dNTPs, and 1 unit of Taq DNA polymerase were performed at 99 °C for 5 s, followed by 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 40 s, for a total of 30 cycles. PCR products were separated by electrophoresis in a 1% agarose gel.

**Densitometry and Quantification—** Western blot analysis of protein extracts was pre-cleared with 50 μl of protein G-agarose overnight at 4 °C. The protein G-agarose/immune complex was washed four times with 1 ml of ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, resuspended in 100 μl of SDS-loading buffer, and heated at 95 °C. DNA was extracted using phenol/chloroform, precipitated using isopropanol, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). One hundred μg of genomic DNA was amplified in 50 μl of reaction buffer containing 10 mM dNTPs, 100 ng of primers (designated P1), and 4 units of Taq DNA polymerase (PerkinElmer Life Sciences) using long distance polymerase chain reaction. Primers selected for identification of eEF1A-2/S1 gene presence were designed as follows: Primers 1 (P1), sense (5’-GACAGAGAAAGAGTAATGGC-3’); 4–24 base pairs (bp)) and antisense (5’-GGCCATCTTTGATTTGTTG-3’), 18814–18897 bp); primers 2 (P2), sense (5’-TAGGGCTTCGTTGGAACG-3’); 15752–15770 bp) and antisense (5’-CTACTCTCCCGAATGCTC-3’), 16204–16187 bp). Long distance PCR reactions were carried out at 95 °C for 3 min, followed by 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, for a total of 30 cycles. Regular PCR reactions in buffer containing 200 μg of genomic DNA, 100 ng of primers (P2), 10 mM dNTPs, and 1 unit of Taq DNA polymerase were performed at 99 °C for 5 s, followed by 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 40 s, for a total of 30 cycles. PCR products were separated by electrophoresis in a 1% agarose gel.

**Characterization of eEF1A-2/S1-specific Antibody—** Because of the 92% amino acid homology between the eEF1A-1 and eEF1A-2/S1 proteins, antibodies to distinguish between them were not available. To resolve this matter, we generated an eEF1A-2/S1-specific antibody (anti-eEF1A-2/S1) by immunizing rabbits with a purified peptide composed of a sequence unique to eEF1A-2/S1, designated CB5 (Fig. 1). In addition, an eEF1A-1-specific antibody (anti-eEF1A-1) was similarly produced elsewhere, using the HT7 peptide with sequence unique to eEF1A-1 (Fig. 1).

**RESULTS**

**Characterization of the eEF1A-2/S1-specific Antibody—** To verify the specificity of these polyclonal antibodies against eEF1A-1 or eEF1A-2/S1 proteins, we subcloned the eEF1A-1 and eEF1A-2/S1 cDNAs in the pGEX-2t vector, resulting in a GST fusion protein, and produced recombinant proteins (referred to as GST-eEF1A-1 and GST-eEF1A-2/S1, respectively). After induction with isopropyl-1-thio-β-D-galactopyranoside,
transferred E. coli bacteria were harvested, and bacterial proteins were analyzed by SDS-polyacrylamide gel electrophoresis. In bacteria transformed with a GST vector alone, the 26-kDa GST protein is induced upon treatment with isopropyl-β-D-galactopyranoside, whereas bacteria possessing the pGEX-eEF1A-1 or pGEX-eEF1A-2/S1 constructs expressed 76-kDa proteins representing the GST-eEF1A-1 and GST-eEF1A-2/S1 fusion proteins, respectively (Fig. 2A). These fusion proteins were further purified by affinity chromatography on Sepharose 4B beads and yielded purified proteins of 26 kDa for GST and 76 kDa for GST-eEF1A-1 and GST-eEF1A-2/S1 (Fig. 2B). The size of both eEF1A-1 and eEF1A-2/S1 proteins after thrombin cleavage of the two recombinant fusion proteins, GST-eEF1A-1 and GST-eEF1A-2/S1, was 50 kDa, consistent with the predicted size of the open reading frame of the cDNA sequence for both proteins.

To characterize the peptide-derived antibody for eEF1A-2/S1 protein, immunoblotting assays were performed to assess its reactivity with the purified GST-eEF1A-2/S1 and absence of reactivity with GST-eEF1A-1. In fact, anti-eEF1A-2/S1 recognizes only the GST-eEF1A-2/S1 fusion protein and does not react with GST-eEF1A-1 or GST proteins (Fig. 2C). The anti-eEF1A-1 antibody recognizes only GST-eEF1A-1 and does not cross-react with either GST or GST-eEF1A-2/S1 proteins (Fig. 2C). Furthermore, we assessed the specificity of our antibodies by competition with soluble HT7 and CB5 peptides (Fig. 2D); the binding capability of anti-eEF1A-1 to the GST-eEF1A-1 fusion protein is competed by soluble HT7 peptide in a concentration-dependent manner, whereas it is unaffected by soluble CB5 peptide (Fig. 2D). Conversely, the CB5 soluble peptide competes for the reaction of anti-eEF1A-2/S1 to GST-eEF1A-2/S1 fusion protein in a concentration-dependent manner, and the HT7 peptide does not compete for anti-eEF1A-2/S1 to GST-eEF1A-2/S1 (Fig. 2E). Therefore, we conclude that the CB5 peptide-derived anti-eEF1A-2/S1 is specific for its intended substrate, eEF1A-2/S1, and the HT7 peptide-derived anti-eEF1A-1 is specific for eEF1A-1.

We used a monoclonal antibody that recognizes both eEF1A-1 and eEF1A-2/S1 in an immunoprecipitation reaction against total protein extracts from adult rat tissues (liver, skeletal muscle, and brain) and determined the presence of eEF1A-1 and eEF1A-2/S1 in the immunoprecipitated products by Western blot analysis, using either anti-eEF1A-1 or anti-eEF1A-2/S1 antibodies. Fig. 3 shows the total and immunoprecipitated protein extracts probed with the anti-eEF1A-1 monoclonal antibody (A), secondary antibody alone as a control (Panel B), and the anti-eEF1A-1 and anti-eEF1A-2/S1 antibodies (C and D, respectively). Evidently the monoclonal antibody for eEF1A-1 immunoprecipitates both eEF1A-1 and eEF1A-2/S1 proteins; probing the immunoprecipitates with this antibody detects the presence of both eEF1A isoforms, eEF1A-1 in liver and eEF1A-2/S1 in skeletal muscle and brain. The presence of eEF1A-1 (which has a slightly smaller molecular weight) was not detected in muscle and brain, because we used adult tissues, where the level of eEF1A-1 protein is drastically diminished in later postdevelopmental stages (described in this report; Figs. 4 and 7). However, eEF1A-1 was detected in liver, an eEF1A-2/S1-negative tissue (Fig. 3A). To verify this finding, we probed the immunoprecipitates with anti-eEF1A-1 and anti-eEF1A-2/S1 antibodies; we found eEF1A-1 only in the liver (and a small amount in the brain (Fig. 3D)) and eEF1A-2/S1 only in skeletal muscle, and the latter as a major band in brain (Fig. 3C). These results verify the specificity of the anti-eEF1A-1 and anti-eEF1A-2/S1 polyclonal antibodies in tissue extracts and demonstrate that the commercially available monoclonal antibody to eEF1A-1 recognizes both isoforms of eEF1A indiscriminately.

"Tissue-specific Presence of eEF1A-2/S1 Protein in Mice, Rats, and Humans—Our laboratory reported previously that eEF1A-2/S1 mRNA expression is restricted to three tissues: brain, heart, and skeletal muscle. The appearance of eEF1A-
Characterization of eEF1A-1 and eEF1A-2/S1 Expression

2/S1 mRNA is a late differentiation event, occurring after myotubes are formed and neuronal terminal differentiation is completed (1). Using anti-eEF1A-1 and anti-eEF1A-2/S1 antibodies, we investigated the presence of eEF1A-1 and eEF1A-2/S1 proteins in various tissues previously characterized for positive or negative expressions of eEF1A-2/S1 mRNA. Immunoblotting analyses were performed on various tissues isolated from adult mice, rats, and humans. A major band of 50 kDa in mouse liver and spleen was detected using the anti-eEF1A-1 antibody (Fig. 4A). The 50-kDa eEF1A-1 protein was also present at a very low level in heart and reduced in abundance in brain and skeletal muscle (Fig. 4A). By probing with anti-eEF1A-2/S1, a 50-kDa protein was detected only in brain, heart, and muscle (Fig. 4B). Anti-eEF1A-2/S1 also detected two other bands at 66 and 45 kDa, to determine whether these two additional bands were specific, a reaction with a rabbit antisemur obtained prior to immunization was compared with the anti-eEF1A-2/S1 reaction, in which the preimmune serum also yielded 66- and 45-kDa protein bands but lacked the specific reaction with the 50-kDa eEF1A-2/S1 protein (Fig. 4C). We conclude that the 66- and 45-kDa bands detected using anti-eEF1A-2/S1 are the result of nonspecific reactions of the preimmune serum.

Similar studies in adult rat (3 months) and human autopsy tissues (68-years-old) revealed the same pattern of expression found in mice. In rat and human tissue, eEF1A-2/S1 protein is restricted to brain, heart, and muscle (Fig. 4, IIA, IIB, IIIA, and IIIB). However, eEF1A-1 protein levels in these three eEF1A-2/S1-positive tissues vary among the three tested species, humans, mice, and rats, ranging from non-detectable to modest levels. This variation may be due to species differences. Nevertheless, results of these studies at the protein level are in overall agreement with the specific gene expression patterns examined at the message level, because eEF1A-2/S1 mRNA is only expressed in brain, heart, and skeletal muscle (3, 6). We also investigated rat smooth muscle (bladder and stomach) for both eEF1A-1 and eEF1A-2/S1 proteins (Fig. 5); lung and spleen were also studied, along with liver and skeletal muscle, which we know are negative and positive, respectively, for eEF1A-2/S1 protein. Fig. 5A shows that eEF1A-1 protein is present in all tested tissues, whereas eEF1A-2/S1 is only present in skeletal muscle but not in smooth muscle of aorta, bladder, or stomach or any other eEF1A-2/S1-negative tissues (including liver, lung, and spleen) (Fig. 5B).

Genotyping of Wild-type and Mutant Mice—The wasted mouse is a spontaneous mutant strain from the Jackson Laboratories (9). The mutant phenotype was recently shown to correlate with a 15.8-kb deletion, including the promoter and first non-coding exon of the eEF1A-2/S1 gene (8) (Fig. 6A). To detect the presence of the mutant allele, we designed oligonucleotide primers to amplify around the boundary of the deletion (the P1 fragment), with the forward primer upstream and the reverse primer downstream, of the 15.8-kb deleted region (Fig. 6A). PCR using these two primers amplifies a 2.9-kb DNA fragment from both homozygous (+/+), heterozygous (+,−) mice (Fig. 6B, lanes 2 and 4). This PCR product is not observed with the wild-type (+/+) mouse DNA, because the expected PCR reaction yields a product larger than 18 kb (Fig. 6B, lane 3). To detect a wild-type allele, oligonucleotide primers were designed to amplify a 456-bp fragment within the 1.5-kb deletion (the P2 fragment). The absence of the fragment was used to identify the homozygous mutants (Fig. 6B, lane 6), whereas the PCR product is readily obtained in the parallel PCR reactions of the wild-type and heterozygous animals (Fig. 6B, lanes 7 and 8).

Presence of eEF1A-1 and eEF1A-2/S1 Proteins in Wild-type, Heterozygous, and wasted Mice—Using anti-eEF1A-1 and anti-eEF1A-2/S1 antibodies, we expanded the previous findings of...
the mRNA levels of both isoforms in wasted mutant mice to a study of their protein presence, detailing the temporal pattern of eEF1A-1 and eEF1A-2/S1 protein levels. In wild-type mice, there is a switch from a predominant eEF1A-1 presence to eEF1A-2/S1 protein in brain, heart, and skeletal muscle during development. eEF1A-1 protein levels are high in embryonic life but gradually decline with postnatal age (Fig. 7). In comparison, the level of eEF1A-2/S1 protein is low in embryonic tissues and increases in abundance up to P28 after birth (Fig. 7, A–C).

Interestingly, some residual eEF1A-1 protein is still detectable in eEF1A-2/S1-positive tissues at P20 and P28. As expected, liver does not express eEF1A-2/S1 protein at any developmental time point (Fig. 7D), consistent with previous reports where eEF1A-2/S1 mRNA was not detected.

The patterns of eEF1A-1 protein presence in heterozygous and wild-type mice are quite similar (Fig. 7). However, in experiments directly comparing eEF1A-1/S1 protein levels in wild-type and heterozygous animals, we detected a decrease in the protein presence of eEF1A-2/S1 in heterozygous animals (data not shown). This was expected, because heterozygous animals lack one eEF1A-2/S1 allele. In homozygous mutant mice we detected no eEF1A-2/S1 protein throughout the entire period of development, up to and including postnatal day 28 (Fig. 7). The absence of eEF1A-2/S1 protein has no effect on eEF1A-1 protein abundance during development in all three tissues that are normally eEF1A-2/S1-positive; the scheduled reduction in eEF1A-1 protein quantity is indistinguishable from that in wild-type mice. Quantitation of eEF1A-1 and eEF1A-2/S1 expression levels in brain, heart, and muscle of wild-type and heterozygous mice reveals an inverse relationship between the expression levels of eEF1A-1 and eEF1A-2/S1. In other words, when eEF1A-1 protein is very abundant, during early embryonic development, eEF1A-2/S1 protein levels are lower and vice versa later. Table I summarizes elongation factor abundance, expressed as a ratio of eEF1A-1 to eEF1A-2/S1.

**Immunofluorescence Microscopy**—Western blot analysis

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**Characterization of eEF1A-1 and eEF1A-2/S1 Expression**

**Table I**

|    | eEF1A-1 | eEF1A-2/S1 | eEF1A-1/eEF1A-2/S1 ratio |
|----|---------|------------|-------------------------|
| **Brain** |          |            |                        |
| E18 | 1.00 ± 0.1 | 0.21 ± 0.03 | 4.76:1 |
| P1  | 0.68 ± 0.15 | 0.45 ± 0.04 | 1.51:1 |
| P7  | 0.55 ± 0.03 | 0.54 ± 0.15 | 1.02:1 |
| P14 | 0.33 ± 0.02 | 1.07 ± 0.42 | 0.31:1 |
| P20 | 0.04 ± 0.01 | 0.98 ± 0.26 | 0.04:1 |
| P28 | 0.01 ± 0.01 | 1.00 ± 0.21 | 0.01:1 |
| **Heart** |          |            |                        |
| E18 | 1.00 ± 0.007 | 0.73 ± 0.050 | 1.37:1 |
| P1  | 0.95 ± 0.007 | 0.90 ± 0.040 | 0.93:1 |
| P7  | 0.50 ± 0.040 | 0.90 ± 0.016 | 0.52:1 |
| P14 | 0.32 ± 0.031 | 0.90 ± 0.029 | 0.32:1 |
| P20 | 0.19 ± 0.025 | 0.99 ± 0.037 | 0.19:1 |
| P28 | 0.07 ± 0.021 | 1.00 ± 0.200 | 0.07:1 |
| **Muscle** |          |            |                        |
| E18 | 1.00 ± 0.031 | 0.16 ± 0.012 | 6.25:1 |
| P1  | 0.95 ± 0.022 | 0.22 ± 0.022 | 4.32:1 |
| P7  | 0.63 ± 0.026 | 0.43 ± 0.034 | 1.47:1 |
| P14 | 0.52 ± 0.036 | 0.81 ± 0.048 | 0.64:1 |
| P20 | 0.32 ± 0.058 | 0.98 ± 0.018 | 0.33:1 |
| P28 | 0.08 ± 0.044 | 1.00 ± 0.012 | 0.08:1 |
| **Liver** |          |            |                        |
| E18 | 1.00 ± 0.21 | ND | ND |
| P1  | 0.36 ± 0.28 | ND | 1.00 ± 0.27 |
| P7  | 0.32 ± 0.19 | ND | 0.32 ± 0.08 |
| P14 | 0.51 ± 0.21 | ND | 0.46 ± 0.11 |
| P20 | 0.42 ± 0.09 | ND | 0.45 ± 0.27 |
| P28 | 0.63 ± 0.05 | ND | 0.54 ± 0.21 |

**ND, not detectable.**
Colocalization of the two proteins in neural cell bodies (yellow, C) and overlaps (L). The solid arrows point to the neural body, which recognizes the eEF1A-2/S1 protein with high affinity. This success, combined with the existing peptide-derived antibodies, allowed us to demonstrate the following. (i) eEF1A-1 protein presence declines at postnatal day 7, with the concomitant decrease of its sister protein, eEF1A-2/S1, in brain, heart, and muscle, where fibroblasts and satellite cells, respectively, may house most of the eEF1A-1 protein, whereas eEF1A-1 is absent in cardiomyocytes and skeletal myotubes, as in neurons.

**DISCUSSION**

We have generated an eEF1A-2/S1-specific polyclonal antibody, which recognizes the eEF1A-2/S1 protein with high affinity. This success, combined with the existing peptide-derived anti-eEF1A-1 (13), provided us two antibodies that distinguish between eEF1A-2/S1 and eEF1A-1 proteins. These antibodies allowed us to demonstrate the following. (i) eEF1A-1 protein presence declines at postnatal day 7, with the concomitant decrease of its sister protein, eEF1A-2/S1, in brain, heart, and skeletal muscle. (ii) wasted mutant mice, as expected, do not express eEF1A-2/S1 protein. (iii) The reduction in eEF1A-1 demonstrates that reactivity against eEF1A-1 in the brain of wild-type mice (20-day-old, P-20) is weak, whereas eEF1A-2/S1 reactivity is intense (Fig. 7A). However, some eEF1A-1 protein is still detectable in the mouse brain, making it unclear whether both isoforms are coexpressed in the same cells. To resolve this matter, we analyzed eEF1A-1 expression by immunohistochemical studies in sections of wild-type mouse brain at postnatal day 20. To assess whether eEF1A-1 expression in brain is contributed by neurons or by non-neuronal cells, we double stained with either anti-eEF1A-1 or anti-eEF1A-2/S1 antibodies, anti-glial fibrillary acidic protein antisera for detection of non-neuronal astrocytes, and an antibody against neurofilament (NF-68). Neurofilament is specifically expressed in neurons, serving as a marker for neuronal localization in brain sections (Fig. 8B, H, K, and N, red). Double staining of wild-type brain sections with anti-eEF1A-2/S1 (Fig. 8A) and anti-NF-68 (Fig. 8B) revealed prominent colocalization of both proteins; merging of the red and green images produced a yellow fluorescence (Fig. 8C), indicating that eEF1A-2/S1 protein is present in the neuronal cells of the brain. We labeled non-neuronal GFAP-positive cells (Fig. 8D) to demonstrate the presence of eEF1A-1 immunoreactivity (Fig. 8E) in the non-neuronal population of the brain sections and found GFAP and eEF1A-1 coimmunoreactivity (Fig. 8F), indicating that both GFAP and eEF1A-1 proteins are localized in the non-neuronal population. As expected, eEF1A-2/S1 protein is not detected in mutant brain (Fig. 8G), although neurons show positive staining with anti-NF-68 in the cell body (Fig. 8, H and I, solid arrows) and dendrites (Fig. 8J, open arrows). On the other hand, brain sections of postnatal day 20 mutant mice reveal similar low level expression patterns of eEF1A-1 in a few cells (Fig. 8J) but strong NF-68 (Fig. 8K), with no obvious colocalization between cells positive for NF-68 (Fig. 8L, open arrows) and those staining weakly for eEF1A-1 (Fig. 8L, solid arrows). Similar to results from mutant mice, double staining wild-type samples with both anti-eEF1A-1 (Fig. 8M, green) and anti-NF-68 (Fig. 8N, red) shows little colocalization (with the exception of two or three neurons with weak eEF1A-1 presence), demonstrating that most eEF1A-1-positive cells have lost this gene expression by this stage of development (Fig. 8O, arrows). These results show that the residual eEF1A-1 presence following the onset of eEF1A-2/S1 appearance is contributed by the non-neuronal cells in the brain or by at most low level presence in neurons. It is likely that analogous situations exist in the heart and muscle, where fibroblasts and satellite cells, respectively, may house most of the eEF1A-1 protein, whereas eEF1A-1 is absent in cardiomyocytes and skeletal myotubes, as in neurons.

**Fig. 8.** Indirect immunofluorescence labeling and confocal micrographs of 20-day-old (P-20) mouse brain. Top panels (A–C) show localization of eEF1A-2/S1 (A, green) and neurofilament (B, red) and colocalization of the two proteins in neural cell bodies (C, yellow, solid arrows) in brains of wild-type mice. D–F, both normal and mutant P-20 brains labeled with anti-GFAP (D, green), anti-eEF1A-1 (E, red), and their overlap (F, yellow) show the same results. G–I show mutant brain labeled with anti-eEF1A-2/S1 (G) and anti-NF-68 (H, red) antibodies and double-stained with both antibodies (I). As expected, the mutant brain shows no eEF1A-2/S1 immunoreactivity (G and I). J–L show wasted mutant brain stained with eEF1A-1 (J, light green) and NF-68 (K, red) and overlaps (L). The solid arrows point to the neural body, which shows both NF-68 and low level eEF1A-1 staining (L), whereas dendrites show only NF-68-positive staining (L, open arrows). M–O show wild-type brain immunolabeled with eEF1A-1 (M, green) and NF-68 (N, red) and colocalization (O, yellow). Note eEF1A-1 and neurofilament colocalization in a few neurons (O, solid arrows), whereas most neurons stain eEF1A-1 negative but NF-68 positive (O, open arrows). The eEF1A-1 positivity in some neurons may suggest that at this stage some neurons still retain residual positivity. The magnification scale of A–O is shown in F, H, and I; the bar is 100 μm.
protein abundance occurs on schedule in wasted mutant mice, as in their wild-type counterparts, indicating that the decline of eEF1A-1 protein abundance is regulated independently of eEF1A-2/S1 protein presence. Therefore, it appears that by postnatal day 28 wasted mutant mice are deficient in two isoforms of peptide elongation factor-1A, which are essential for normal protein synthesis.

During the course of development of the wasted mutant mouse, we failed to detect eEF1A-2/S1 protein at any postnatal stage, which is in agreement with the absence of eEF1A-2/S1 mRNA in mutant mice (8). It is important to note that the absence of eEF1A-2/S1 protein is not compensated for by any increase in eEF1A-1, in agreement with Chambers et al. (8), who detected no substantial level of eEF1A-1 mRNA in the brain, heart, or muscle of wasted mice. Therefore, these tissues in the wasted mutant do not express any known peptide elongation factor-1A; so it is to be expected that there is a deficiency of protein synthesis in these tissues. However, no reports so far describe protein synthesis in wasted mice. In the mouse brain, a residual amount of eEF1A-1 remains even in adulthood, in contrast with heart and muscle, where its expression is almost undetectable. It is well known that the brain has a very heterogeneous cellular composition; thus, to determine whether eEF1A-1 expression is found in neurons, where eEF1A-2/S1 is expressed, immunohistochemical studies were performed. We demonstrate that the residual expression of eEF1A-1 in the adult brain is attributed mostly to non-neuronal cells, suggesting that only neurons express eEF1A-2/S1 as a peptide elongation factor-1A, which is consistent with the previous report of Lee et al. (19).

Taken together, our findings favor the possibility that the cause of death in wasted mutant mice may be a drastic reduction or absence of peptide elongation factor-1A. Despite the absence of eEF1A-2/S1 in the wasted mutant mice, the possibility that other mutations coexist with the eEF1A-2/S1 deletion cannot be excluded, as argued by Chambers et al. (8). Therefore, the phenotype of the wasted mutant may result from additional mutations. In addition to muscle wasting and motor neuron loss, the wasted phenotype is also associated with immune system abnormalities and defects in mechanisms of DNA repair (20), where the presence and role of eEF1A-2/S1 would be obscure.

There are many possible functional implications for a shift in expression from eEF1A-1 to eEF1A-2/S1 in brain, heart, and skeletal muscle during development. It is reported that eEF1A-2/S1 performs peptide elongation in vitro, with the only known difference being a slower rate of GDP dissociation (7), which may result in increased translational fidelity. Indeed, a correlation between translational accuracy and GTP hydrolysis has been reported (21–24). It is also possible that eEF1A-2/S1 retains the protein elongation role of eEF1A-1 but not all of its other functions, most importantly the bundling of actin filaments (25) and severing of microtubules (26). Investigation into the peptide domains responsible for the shared protein elongation function and for the dissimilar non-canonical functions between the two sister eEF1As will definitively explain the development-dependent shift in expression of these two genes. Nonetheless, our results show that in neurons, myotube and cardiomyocytes, eEF1A-1 is the embryonic form of peptide elongation factor, whereas its sister, eEF1A-2/S1, is the adult form.

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REFERENCES

1. Lee, S., Wolfram, L. A., and Wang, E. (1993) J. Biol. Chem. 268, 24453–24459
2. Merrick, W. C. (1992) Microbiol. Rev. 56, 291–315
3. Lee, S., Franceau, A. M., Liu, S., and Wang, E. (1992) J. Biol. Chem. 267, 24064–24068
4. Lee, S., Duttaroy, A., and Wang, E. (1996) in Cellular Aging and Cell Death, pp. 139–151. Wiley-Liss, Inc., New York
5. Ann, D. K., Moutsatsos, I. K., Nakamura, T., Lin, H. H., Mao, P. L., Lee, M. J., Chin, S., Liem, R. K., and Wang, E. (1991) J. Biol. Chem. 266, 10429–10437
6. Knudsen, S. M., Frydenberg, J., Clark, B. F., and Leffers, H. (1993) Eur. J. Biochem. 215, 549–554
7. Kahns, S., Lund, A., Kristensen, P., Knudsen, C. R., Clark, B. F., Cavallius, J., and Merrick, W. C. (1998) Nucleic Acids Res. 26, 1884–1890
8. Chambers, D. M., Peters, J., and Abbott, C. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4463–4468
9. Shultz, L. D., Sweet, H. O., Davissson, M. T., and Coman, D. R. (1982) Nature 297, 402–404
10. Lutsep, H. L., and Rodriguez, M. (1989) J. Neuropathol. Exp. Neurol. 48, 519–533
11. Inoue, T., Tezuka, H., Kada, T., Aikawa, K., and Shultz, L. D. (1986) Basic Life Sci. 39, 323–335
12. Welle, S., Thornton, C., Bhatt, K., and Kryms, M. (1997) J. Gerontol. A Biol. Sci. Med. Sci. 52, B235–B239
13. Liu, C. H., Liu, S., and Wang, E. (1993) Biochem. Biophys. Res. Commun. 195, 1371–1378
14. Upreti, G. C., Ratcliffe, R. A., and Riches, P. C. (1988) Anal. Biochem. 168, 421–427
15. Smith, D. B., and Johnson, K. S. (1988) Gene 67, 31–40
16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Kaur, J., and Ruben, L. (1994) J. Biol. Chem. 269, 23045–23050
18. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991) Nucleic Acids Res. 19, 4253
19. Lee, S., LeBlanc, A., Duttaroy, A., and Wang, E. (1995) Exp. Cell Res. 219, 589–597
20. Inoue, T., Aikawa, K., Tezuka, H., Kada, T., and Shultz, L. D. (1986) Cancer Res. 46, 3979–3982
21. Thompson, R. C., and Karim, A. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4922–4926
22. Carr-Schmid, A., Durko, N., Cavallius, J., Merrick, W. C., and Kinay, T. G. (1999) J. Biol. Chem. 274, 30327–30332
23. Carr-Schmid, A., Valente, L., Leik, V., I., Williama, T., Starita, L. M., and Kinay, T. G. (1999) Mol. Cell. Biol. 19, 5257–5266
24. Gopalkrishnan, R. V., Su, Z. Z., Goldstein, N. I., and Fisher, P. B. (1999) Int. J. Biochem. Cell Biol. 31, 151–162
25. Yang, F., Demma, M., Warren, Y., Dharmawardhane, S., and Condeelis, J. (1991) Nature 347, 494–496
26. Shiina, N., Getoh, Y., Kubomura, N., Iwamatsu, A., and Nishida, E. (1994) Science 266, 282–285