A cDNA from rat skeletal muscle encoding calcium/calmodulin-dependent eukaryotic elongation factor-2 kinase (eEF-2K) has been cloned and sequenced, and the amino acid sequence of the protein has been deduced. The kinase is composed of 724 amino acids and has a predicted molecular mass of 81,499 Da. The cDNA was judged to be full-length, as the protein, expressed in rabbit reticulocyte lysate or wheat germ extract, migrated upon SDS-PAGE with the same apparent molecular weight as the purified kinase and possessed eEF-2K activity. eEF-2K contains all of the 12 catalytic subdomains present in the majority of protein kinases, but they are atypical and display only limited homology with other kinases. A putative calmodulin-binding domain is present C-terminal to the catalytic domain as is a putative pseudosubstrate sequence. Two antipeptide antibodies raised against sequences derived from a partial rabbit cDNA clone, cross-reacted with purified eEF-2K, and one also immunoprecipitated eEF-2K activity from cell extracts. Northern blot analysis demonstrated that eEF-2K mRNA is expressed in a number of different tissues and that it may exist in multiple forms.

Cloning and Expression of cDNA Encoding Protein Synthesis Elongation Factor-2 Kinase

(Received for publication, December 20, 1995, and in revised form, March 28, 1996)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) X96426.

† This work was supported by a postdoctoral fellowship from the Wellcome Trust (to N. T. R.) and by group support from the British Diabetic Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: CaM, calmodulin; CaM-PK, Ca2+/CaM-dependent protein kinase; eEF-2K, eukaryotic elongation factor-2 kinase; FSBA, fluorosulfonylbenzoylalanine; GTP, guanosine triphosphate; HEDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; PABA, p-aminobenzoic acid; PAGE, polyacrylamide gel electrophoresis; Tricine, N[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction; Mops, 4-morpholinepropanesulfonic acid; bp, base pair(s); kb, kilobase pair(s); UTR, untranslated region; HEDTA, N-hydroxyethylglycine; MOPS, 4-morpholinepropanesulfonic acid.

The enzyme undergoes Ca2+/CaM-dependent intramolecular autophosphorylation, and this results in it becoming partially Ca2+-independent (Redpath and Proud, 1993a; Mitsui et al., 1993), a property shared with the type II (multifunctional) CaM-PK (Hanson and Schulman, 1992a). Autophosphorylation occurs on multiple sites involving both Ser and Thr residues. eEF-2K has been tested in vitro, by cAMP-dependent protein kinase (Redpath and Proud, 1993b; Mitsui et al., 1993). This also renders the enzyme partially Ca2+-independent (Redpath and Proud, 1993b). Thus, eEF-2K can potentially be activated by two different intracellular messenger systems, calcium ions and cAMP, and therefore has the potential to mediate the effects of either on the translational machinery of the cell, resulting in each case in inhibition of peptide chain elongation (see Redpath and Proud (1994) for a recent review). eEF-2K is likely to play a key role in the control of peptide chain elongation by hormones and growth factors. Indeed, it has been shown that agents that raise intracellular calcium ion concentrations lead to increased phosphorylation of eEF-2 (Hincke and Nairn, 1992; Demolle et al., 1990; Palfrey et al., 1987; Mackie et al., 1989).

We now report the cloning and expression of a cDNA encoding rat eEF-2K. Analysis reveals that it is a novel protein kinase, only rather distantly related to other CaM-PKs.
EXPERIMENTAL PROCEDURES

Materials—Chemicals, biochemicals, and other items were obtained as described previously (Redpath and Proud, 1993a). All radiolabels were from Du Pont NEN except (γ-³²P]ATP, which was from Amersham (Buckinghamshire, United Kingdom) as were the Multiprime DNA-labeling kit and ECL immunodetection kit. The Sequenase version 2.0 DNA sequencing kit was obtained from Upstate Biotechnology, Inc./Life Technologies, Inc. cDNA libraries were obtained from Clontech and Stratagene. Restriction enzymes and general molecular biology reagents were from Boehringer Mannheim. Nonradioactive-coated nylon membranes for library screening were obtained from Sartorius. T3/T7-coupled T7 expression kits were obtained from Promega, and the pCRII TA cloning kit was from Invitrogen.

Rabbit reticulocyte eEF-2 was purified as described previously (Redpath and Proud, 1993a), and eEF-2K was purified as described (Redpath and Proud, 1993a) with successive chromatography on S-Phase Sepharose, DEAE-cellulose, phenyl-Sepharose, CaM-Sepharose, and Mono-Q.

Sequencing of Peptides Derived from eEF-2K—Rabbit reticulocyte eEF-2K (0.1 mg) (low molecular mass form (95 kDa)) (Redpath and Proud, 1993a) was subjected to electrophoresis on a 15% SDS-polyacrylamide gel, and the gel chips containing the kinase were excised. CNBr digestion of the gel chips, Tricine gel electrophoresis, and Western blotting were carried out as described (Price et al., 1994). The membrane was stained and destained, and regions containing stained peptides were excised and subjected to peptide sequencing by solid phase automated Edman degradation using an Applied Biosystems 477A sequencer.

Cloning of eEF-2K cDNA—From the obtained peptide sequences, various oligonucleotides were designed for use in PCR. Two of them had the sequences ATGTGGATG(T/C/A/JAT(T/C/G/A/G/G/T/G/T/J/T/G/G/A)-AC (sense encoding MIIWIEL(T)) and GT(T/C/J/C/T/G/G/G/T/G/J/A/T/G/A/G/A(T/C/G/G/T/G/J/G/G/A/G/G/T/G/J/T/G/G/T/G/J/T/G/G/A/A (antisense corresponding QDHLDN(Q)); the parentheses indicate that only the first two bases of the triplets that code for these amino acids were used). PCR conditions were 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C for 35 cycles followed by 72 °C for 10 min. PCR products were gel-purified before cloning into pUC18 or pCR1. cDNA libraries were screened according to the manufacturer’s instructions. Membranes were probed with cDNA (labeled with [α-³²P]dCTP by random priming) at 42 °C overnight in 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml sheared salmon sperm DNA. 1 × SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 1 × Denhardt’s solution is 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.15 polyvinylpyrrolidone. After incubation, the membranes were washed twice with 2 × SSC, 0.05% SDS at 55 °C. Positive clones were purified by two further rounds of screening. The membranes from the tertiary screen were washed twice with 2 × SSC, 0.05% SDS at room temperature and twice with 1 × SSC, 0.1% SDS at 55 °C; the cloned cDNAs were excised into pBluescript II SK (Stratagene) as per the manufacturer’s instructions. The largest of the cDNA clones were chosen for DNA sequencing. Manual sequencing in both directions was carried out using the Sanger dideoxy termination method using a Sequenase version 2.0 kit, and parts of the sequence were verified by automatic sequencing. Sequence alignments were made using the LASERGENE suite of programs (from DNASTAR).

Assay of eEF-2K—eEF-2K was assayed in buffer containing 50 mM Mops (pH 7.2), 11 mM MgCl₂, 0.2 mM ATP, 100 μM CTP (γ-³²P]ATP, 5 mM diithiothreitol, 10% glycerol, 0.5 mM benzamidine, 1 μg/ml each of pepstatin, antipain, and leupeptin, 0.4 mM EDTA, 2 mM HEDTA, and 150 pmol of eEF-2 in a final volume of 30 μl for 30 min at 30 °C. CaCl₂ (0.67 mM) and CaM (2 mM) were added as required. Samples were then analyzed by SDS-PAGE and autoradiography. Immunoprecipitation of eEF-2K was performed using 5 mg of protein A-Sepharose and a 1:2.5 dilution of antisera in a final volume of 0.2 ml. The mixture was tumbled at 4 °C for 2 h followed by extensive washing with buffer (50 mM Hepes, pH 7.5, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 30 mM sodium pyrophosphate, 2.5 mM benzamidine, 1 μg/ml each of pepstatin, antipain, and leupeptin, 10 μM EDTA, and 1% Triton X-100) and then with a 1:10 dilution of the buffer before resuspension of the beads in 1:10 diluted buffer. The resuspended beads were used in the assay for eEF-2K as above.

In Vitro Expression of eEF-2K cDNA—cDNA in pBluescript was expressed in reticulocyte lysate and wheat germ extract in the presence of [³⁵S]methionine/cysteine using the T3/T7-coupled Tnt systems according to the manufacturer’s instructions.

Preparation of Antipeptide Antibodies—Two peptides, CLRLSENS-GDENMSDV(T) (p1) and CDLSPREDAQVNTKTL (p2), were synthesized and linked to keyhole limpet hemocyanin (van Regenmortel et al., 1988). Antibodies were raised in New Zealand White rabbits. The peptides were sequences that were deduced from the sequence of the 663-bp PCR product, amplified from rabbit cDNA (see “Results and Discussion”).

Northern Blot Analysis—Poly(A)⁺ RNA was prepared from 0.5 mg of total RNA from various rat tissues as described (Craddock et al., 1995). This was separated on a formaldehyde-containing gel and blotted onto Hybond-N membrane. eEF-2K mRNA was detected using a probe derived from an EcoRI restriction product from eEF-2K cDNA corresponding to bases 589-2681. Blots were incubated overnight at 42 °C in 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 0.5% SDS, and 0.2 mg/ml sheared salmon sperm DNA followed by washing with 2 × SSC, 0.01% SDS three times at room temperature and with 1 × SSC, 0.1% SDS three times at 65 °C. The blot was stripped by washing extensively in 0.01 × SSC, 1% SDS at 90 °C and then reprobed for β-actin.

Labeling of Proteins with [¹⁴C]Fluorosulfonylbenzoyladenosine—Labeling was performed in 20-μl incubations containing 5 mM MgCl₂, 50 μM CaCl₂, 2 μg/ml CaM, 0.04 μCi of FSBA. Where required, 200 μM ATP was also added. As FSBA binds to the ATP-binding site, ATP should act as a competitive inhibitor of labeling. Incubations were for 25 min at 30 °C.

RESULTS AND DISCUSSION

Characterization of the Low and High Molecular Weight Forms of eEF-2K—As previously reported (Redpath and Proud, 1993a; Mitsui et al., 1993), purification of eEF-2K often results in the co-isolation of two polypeptides, differing in apparent molecular mass by about 10 kDa. We previously concluded that both possess an ATP-binding site. Furthermore, no other proteins are labeled in these highly purified kinase preparations. This demonstrates that the 100-kDa proteins do not associate or co-purify with another protein that binds ATP and might thus possess eEF-2 kinase activity. Second, antisera directed against two peptide sequences deduced from a partial eEF-2K cDNA clone (see below) cross-react with both forms of eEF-2K (5 pmol, lanes 3 and 4), and PKA (33 pmol, lanes 5 and 6) were labeled with [¹⁴C]FSBA as described under “Experimental Procedures” in the absence or presence of 200 μM ATP as indicated. PKA was used as a positive control. Gels were processed for fluorography and imaged using a ¹⁴C-sensitive screen. The arrows on the left indicate the high and low Mw, weight forms of eEF-2K, whereas the arrow on the right indicates PKA.

FIG. 1. Labeling of eEF-2K and PKA with FSBA. Highly purified high Mw, weight form eEF-2K (6 pmol, lanes 1 and 2), low Mw, weight form of eEF-2K (5 pmol, lanes 3 and 4), and PKA (33 pmol, lanes 5 and 6) were labeled with [¹⁴C]FSBA as described under “Experimental Procedures” in the absence or presence of 200 μM ATP as indicated. PKA was used as a positive control. Gels were processed for fluorography and imaged using a ¹⁴C-sensitive screen. The arrows on the left indicate the high and low Mw, weight forms of eEF-2K, whereas the arrow on the right indicates PKA.
species, and this clone contained all of the amino acid sequences obtained from peptides derived from the 95-kDa protein. Although it cannot be completely ruled out that the two forms of eEF-2 kinase could result from two alleles or two closely related genes, it seems highly likely that the 95-kDa protein is produced by proteolytic nicking of the larger species, particularly considering that different preparations of kinase differ in the relative amounts of the two forms of the kinase (see Redpath and Proud (1993)). It seems unlikely that the two forms result from alternative initiation sites, since expression of the cDNA in rabbit reticulocyte lysate (see below) results in the synthesis of only the higher molecular weight form of the kinase. In this context it should be noted that while the larger polypeptide has a blocked N terminus, the smaller one's amino terminus was not blocked (data not shown), suggesting that the proteolytic modification includes removal of N-terminal peptides.

Peptide Sequence Data for eEF-2K—The peptide sequences obtained were used to design redundant oligonucleotide primers. Use of two of the primers whose sequences are given under “Experimental Procedures” in the PCR amplified a single product (663 bp) when rabbit reticulocyte cDNA was used as the template. This was sequenced and shown to contain sequences corresponding to peptide B as well as peptides C and D, from which the PCR primers were derived. The 663-bp cDNA was used to screen an oligo(dT)-primed rabbit liver cDNA library (Stratagene) and a rabbit reticulocyte cDNA library (provided by J.-J. Chen, Massachusetts Institute of Technology) with the 663-bp cDNA did not result in the identification of any positive clones.

Isolation of cDNAs Encoding eEF-2K—The peptide sequences obtained were used to design redundant oligonucleotide primers. Use of two of the primers whose sequences are given under “Experimental Procedures” in the PCR amplified a single product (663 bp) when rabbit reticulocyte cDNA was used as the template. This was sequenced and shown to contain sequences corresponding to peptide B as well as peptides C and D, from which the PCR primers were derived. The 663-bp cDNA was used to screen an oligo(dT)-primed rabbit liver cDNA library (Stratagene) and a rabbit reticulocyte cDNA library (provided by J.-J. Chen, Massachusetts Institute of Technology) with the 663-bp cDNA did not result in the identification of any positive clones.

The 1.3-kb cDNA was then used as a probe to screen a rat skeletal muscle cDNA library in λgt11 (Clontech). One positive clone was obtained from this screen with an insert size of approximately 1.3 kb. Sequencing of this showed that it contained the entire sequence of the 663-bp cDNA apart from its 5'-terminal 76 nucleotides. Screening of a rat liver cDNA library (Stratagene) and a rabbit reticulocyte cDNA library (provided by J.-J. Chen, Massachusetts Institute of Technology) with the 663-bp cDNA did not result in the identification of any positive clones.

The 1.3-kb cDNA was then used as a probe to screen a rat skeletal muscle cDNA library in λgt11 (Clontech). Approximately 53 positive plaques were obtained from a screen of about 1.5 × 10⁶ plaque-forming units. The cDNA and deduced amino acid sequences of the largest clone are shown in Fig. 3A. The clone consists of 4800 nucleotides having a 290-nucleotide 5'-untranslated region (UTR), a 2175-nucleotide coding region (including the termination codon), encoding 724 amino acids, and a 2335-nucleotide 3'-UTR. The encoded eEF-2K has a predicted molecular mass of 81,499 Da and an isoelectric point of 5.0. The predicted molecular weight is somewhat lower than the molecular weight of the kinase as judged by SDS-PAGE (100 kDa) (Redpath and Proud, 1993a). Apparent anomalies of this kind, however, are not uncommon. For example, the eukaryotic initiation factor-2a kinase (heme-controlled repressor) migrates as a 90-kDa protein upon SDS-PAGE, but it has a predicted molecular mass of only 70 kDa (Chen et al., 1991). It is possible that the highly acidic N-terminal region of eEF-2K is responsible for its anomalous behavior upon SDS-PAGE; 31% of the first 35 residues are either glutamate or aspartate.

All four peptides obtained by sequencing could be identified in the cloned rat sequence, although peptide A was only a poor match that is likely to reflect differences between the sequences of the rat and rabbit proteins.

The sequence around the initiation codon (nucleotides 291-293) fits very well with the consensus sequence (GCCGCC- (A/G)CCAUGG) (Kozak, 1989), the most important nucleotides

| Peptide | Sequence |
|---------|----------|
| A       | (M)AREQDHLDONGREDSDN |
| B       | (M)GLAPFDLSPRQKLAVNO |
| C       | (M)WIILERTPGRPLHILEHYI |
| D       | (M)REGFRKLSFLHIAAGQKK |
Cloning of Elongation Factor-2 Kinase

Fig. 3. Sequence of rat skeletal muscle eEF-2K cDNA and deduced amino acid sequence. A, the underlined sequences indicate the four peptides obtained by peptide sequencing. The discrepancies between the peptide sequences (from the rabbit protein) and the sequence deduced from the rat cDNA are likely to be due to species differences. The numbers on the left refer to the nucleotide sequence, whereas those on the right refer to the amino acid sequence. The star denotes the termination codon. Also indicated is the putative calmodulin-binding domain (double underlined). The putative pseudosubstrate sequence is located between residues 609 and 626 and is boxed. B, the domain structure of eEF-2K is shown. RD indicates the regulatory domain, which contains the putative CaM-binding and autoinhibitory domains. The figure is drawn to scale. C, residues 595–612 of eEF-2K are presented as a right-handed α-helical wheel. The hydrophobic and basic faces are indicated. D, the putative pseudosubstrate sequence in eEF-2K is compared with the region containing the phosphorylation sites in eEF-2. The main phosphorylation site in eEF-2 is Thr56. The solid lines indicate sequence identity, whereas the dashed lines indicate residues of similar character.
site, thus excluding the substrate. Examples of such kinases include CaM-PKs I (Yokokura et al., 1995), II (Brickey et al., 1994), and IV (Tokumitsu et al., 1994), myosin light chain kinase (Kreuger, et al., 1995) and p70S6k (see Banerjee et al. (1990)). In the case of the CaM-PKs, the binding of Ca$^{2+}$/CaM activates the kinase by removing the autoinhibitory domain from the active site. In these protein kinases, the sequence within the autoinhibitory domain is often similar to that surrounding the phosphorylation site in the substrate. Residues 609–626 in eEF-2K are rather similar to the region surrounding the main phosphorylation site in eEF-2 (Thr56). Alignment of residues 609–626 of eEF-2K with residues 41–58 of eEF-2 (Fig. 3D) shows that these regions have 28% identity and 67% homology. The equivalent of the main phosphorylated residue in eEF-2 (Thr56) is Asn624 in eEF-2K.

Examination of the amino acid sequence of eEF-2K reveals a surprisingly low homology with other serine/threonine protein kinases. The vast majority of kinases whose sequences are known possess 12 catalytic subdomains that contain a number of highly conserved residues, most of which are involved in binding Mg$^{2+}$/ATP (see Hanks and Hunter (1995)). Fig. 4 shows the conserved consensus sequence of the catalytic domain of cAMP-dependent protein kinase (PKA) compared with the subdomains of eEF-2K. PKA fits well with the consensus sequence, as do the other CaM-PKs (Hanks and Hunter, 1995).

In subdomain I, eEF-2K contains the GXGXXG motif, which is conserved in ATP-binding proteins but lacks the nearly invariant valine two residues downstream of this sequence, which contributes to a hydrophobic pocket. Serine 53 in PKA makes a hydrogen bond with ATP $\beta\gamma$-phosphate oxygens. The equivalent in eEF-2K (Asp436) should also be able to make a hydrogen bond. Although eEF-2K has a usually conserved hydrophobic residue (Phe) immediately before the first glycine, it does not have the usually conserved hydrophobic residue seven residues before the first glycine (this is Thr in eEF-2K). The next lysine in the eEF-2K sequence is at position 340 and is likely to be the invariant lysine in subdomain II. This residue appears to be essential for the binding of ATP, as it interacts with the $\alpha$- and $\beta$-phosphates (Hanks and Hunter, 1995). Alanine 70 (in PKA) contributes to the hydrophobic adenine ring pocket, but this is replaced in eEF-2K by Ser338. Subdomain V is important in linking the small and large lobes of the catalytic domain in PKA. Residues important in this appear to be Glu121, Val123, and Glu126. These are generally poorly conserved in eEF-2K. However, three residues that contribute to the hydrophobic pocket in PKA (Met120, Tyr122, and Val123) are conservedly replaced by Pro412, Phe414, and Gly415 in eEF-2K.

The most notable difference between the sequences of eEF-2K and PKA occurs in subdomain VIb. The conserved aspartate residue (Asp156 in PKA, Asp431 in eEF-2K) is vital in...
kinase activity, since this residue seems to accept a proton from the attacking hydroxyl group. The near-invariant lysine (Lys168 in PKA) appears to be involved in neutralizing γ-phosphate negative charge. This is replaced by Glu435 in eEF-2K, which obviously cannot carry out this function. Furthermore, eEF-2K does not have the highly conserved asparagine in this subdomain (Asn171 in PKA). Subdomain VII in most protein kinases contains a highly conserved DFG sequence, which is DSG in eEF-2K. This means that subdomains VIIb and VII in eEF-2K are relatively close together in the primary sequence, the more usual spacing being at least 19 residues. Asp184 and Gly186 (in PKA) form hydrogen bonds to stabilize a loop, and Asp186 also chelates Mg\(^{2+}\) that bridges the β- and γ-phosphates.

The highly conserved motif in subdomain VIII is PE, almost always APE or SPE. In eEF-2K this is DPE. Glu208 (in PKA) forms an ion pair with the conserved arginine in subdomain XI, stabilizing the large lobe. Subdomain IX contains a highly conserved DXXXXG motif, within which there are almost always several hydrophobic residues. This subdomain in PKA may play a major role in peptide substrate recognition and therefore may not be expected to show a high degree of conservation with eEF-2K. This motif, however, is present in eEF-2K, although it lacks the conserved highly hydrophobic nature of this motif. This sequence in eEF-2K is, however, rather similar to the corresponding sequence in casein kinase-1α (DMESLG).

In summary, although the sequence of the catalytic domain of eEF-2K shows surprisingly low conservation with the consensus sequence and the other CaM-PKs, almost all of the essential residues are present in eEF-2K, with the exception of the highly conserved lysine and asparagine residues in subdomain VIIb. Molecular modeling will be required to determine how the three-dimensional structure of the catalytic domain of eEF-2K compares with those of the other protein kinases.

We have previously shown that rabbit eEF-2K is a substrate for PKA (Redpath and Proud, 1993b). PKA generally phosphorylates Ser/Thr residues with basic residues N-terminal to the target residue, usually RRS(T/S) (Hanks and Hunter, 1995). There are no perfect matches to this consensus in rat eEF-2K, but there are several approximate fits, i.e. KKL (residues 149–152), KRS (446–448), and RRPES (465–469). There are also a number of Ser/Thr-Pro motifs, which are potential phosphorylation sites for "proline-directed" kinases such as mitogen-activated protein kinase. Further work is required to determine whether eEF-2K can indeed be phosphorylated by such enzymes and whether this modulates its activity. The availability of the primary sequence of eEF-2K will be a very valuable aid in the identification of the sites in eEF-2K phosphorylated by PKA, by autophosphorylation and, where relevant, other protein kinases.

Expression of cDNA Encoding eEF-2K—The cDNA was expressed by coupled transcription/translation in the reticulocyte lysate system in the presence of \(^{35}S\)-labeled amino acids. A single radiolabeled band of about 100 kDa, which was present only in the lysate supplemented with cDNA, was observed (Fig. 5). This corresponds closely to the molecular weight of the purified kinase from rabbit reticulocytes (Redpath and Proud, 1993a; Mitsui et al., 1993). Attempts were made to detect an increase in eEF-2K activity associated with expression of this protein relative to the endogenous activity present in the lysate. However, no detectable increase was apparent above the endogenous level, which is already quite substantial in reticulocyte lysates.

We therefore turned to the wheat germ system, which is reported to be devoid of endogenous eEF-2K (Smailov et al., 1993). Transcription/translation in the presence of \(^{35}S\)-labeled
methionine/cysteine gave low levels of a labeled band at 100 kDa, but the efficiency of expression was much lower than in the reticulocyte lysate system. Therefore, only a low level of eEF-2K activity was detected in samples that contained the eEF-2K cDNA, which was absent from the minus cDNA control (not shown). In order to eliminate the interfering background phosphorylation due to endogenous proteins in the wheat germ lysate, we made use of anti-eEF-2K peptide antibodies. The antiserum raised against peptide 1 immunoprecipitated eEF-2K activity from the wheat germ system supplemented with the cDNA but not from an appropriate control lacking the eEF-2K cDNA (Fig. 6A). Furthermore, the kinase expressed in wheat germ extract was fully dependent on Ca\(^{2+}\)/CaM for activity (Fig. 6B). These data demonstrate conclusively that the cDNA does indeed encode eEF-2K.

Homology of eEF-2K with Other Proteins—A search of a protein sequence data base (SwissProt) revealed no significant homology of eEF-2K with any other mammalian protein. The most highly homologous sequence (37% identity) was from Caenorhabditis elegans (Fig. 7). This sequence is a putative open reading frame in the F-42A10.4 gene sequence (cosmid accession number U10414) and was obtained from the C. elegans genome-sequencing project (see Wilson et al. (1994)), and it is reported as having highest homology with ATP-binding transport proteins. Its relationship with eEF-2K is unknown, but it is possible that it is the C. elegans homologue of eEF-2K. However, the fact that the region of highest homology occurs outside the catalytic domain of eEF-2K may argue against this. The only other protein to show any significant homology with eEF-2K was Dictyostelium myosin heavy chain kinase (GenBank\(^{TM}\) accession number P42527) (not shown).

Northern Blot Analysis of eEF-2K mRNA—Probing of poly(A)\(^{+}\) RNA for eEF-2K revealed the presence of three or four labeled species that were all expressed in the tissues tested (Fig. 8). The most abundant band ran at about 2.5–3 kb, while the larger species were 4–5 kb. This suggests that eEF-2K mRNA may exist mainly in a form considerably smaller than the cDNA doned here. Alternatively, the long 3' UTR may have been an artifact produced during the construction of the cDNA library such as the fusion of unrelated sequence to the 3' UTR of eEF-2K. However, a database search for homology to the 3' UTR of eEF-2K did not reveal any identity with other sequences. In addition, PCR was used to show whether a product corresponding to the size of the 3' UTR could be amplified from rat skeletal muscle cDNA. Nonredundant primers complimentary to the 3' end of the coding region and the 3' end of the 3' UTR were used, and an approximately 2.4-kb cDNA was amplified (not shown). This demonstrates that eEF-2K mRNA species with very long 3' UTRs do exist. Furthermore, an approximate 2.6-kb cDNA clone was isolated, the 3' UTR of which was only 113 bases, ended in a poly(A) tail, and was identical to the first 113 bases of the 3' UTR of the clone shown in Fig. 3A. It is therefore very likely that the long 3' UTR of the clone shown here results from incomplete processing and that the completely processed mRNA is about 2.6 kb, consistent with the results obtained from Northern analysis. The 2.6-kb clone also had an identical 5' UTR to the clone shown here, indicating that the 5' UTR is authentic and not artifically produced during construction of the library. Finally, it should be noted that the largest clone obtained was chosen for sequence analysis. Of seven clones isolated, only two were larger than 2.6 kb, indicating that eEF-2K mRNA transcripts larger than 2.6 kb are a minor species, again consistent with the Northern analysis shown here.

eEF-2K mRNA is expressed at high levels in skeletal muscle in comparison with the other tissues tested, and the relative levels of mRNA are in general agreement with the relative amount of eEF-2K activity that was measured in various rat tissues by Nairn et al. (1985). The relative eEF-2K activity in skeletal muscle, liver, heart, and cerebellum was found to be 1:0.2:0.1:0.08, respectively (Nairn et al., 1985).

General Discussion—The data presented here detail the first cloning of cDNA encoding eEF-2K and deduction of the amino acid sequence. Several pieces of evidence prove that the cloned cDNA does indeed encode eEF-2K. Most compellingly, the expressed protein has the same molecular weight as the purified kinase and possesses Ca\(^{2+}\)/CaM-dependent eEF-2K activity. Furthermore, all of the peptide sequences obtained by sequencing of purified kinase were found in the protein sequence de-
duced from the cDNA, and antibodies raised against peptide sequences within the kinase cross-react with purified eEF-2K and immunoprecipitate eEF-2K activity from cell extracts.

Perhaps the most surprising finding resulting from the determination of the primary sequence of eEF-2K is the relatively low homology of the catalytic domains with other serine/threonine protein kinases. There are other proteins that possess protein kinase activity but which have kinase domains completely unrelated to any eukaryotic or prokaryotic kinase (e.g. Bcr (Maru and Witte, 1991) and “A6” kinase (Beeler et al., 1994)), and compared with them eEF-2K is much closer to the norm. Nonetheless, it is surprising that the catalytic subdomains of eEF-2K do not more closely resemble those of the other protein kinases, particularly the CaM-PKs. Cloning of eEF-2K from other species will identify the relationship of eEF-2K from different species and which residues are highly conserved and, by implication, important for the function of eEF-2K.

The identification of regions of eEF-2K as CaM-binding and autoinhibitory domains as discussed above is at this stage speculative. Where they have been identified, the CaM-binding and autoinhibitory domains in the other CaM-PKs lie close to each other. In CaM-PKs I, II, and IV and in myosin light chain kinase, the autoinhibitory domain is proposed to be N-terminal to, but overlapping with, the CaM-binding domain (Kreuger et al., 1995; Tokumitsu et al., 1994; Hanson and Schulman, 1992b; Yokokura et al., 1995). Mutagenesis studies should help to identify which residues are important in the CaM binding and autoinhibition of eEF-2K. These studies are currently under way.

Acknowledgments—We thank Lee Clarke for carrying out the initial characterization of the anti-eEF-2K antisera and Emily Foulstone for the gift of anti-p70S6k. We also thank Dr. Peter Martin (International Blood Group Reference Laboratory, Southmead Hospital, Bristol, UK) for carrying out some DNA sequencing.

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