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Cloning of the mitogen-activated S6 kinase from rat liver reveals an enzyme of the second messenger subfamily

(polymerase chain reaction/multiple mRNAs/SDS/PAGE/dephosphorylation)

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ABSTRACT **Recently we reported the purification of a mitogen-activated S6 kinase from Swiss mouse 3T3 fibroblasts and rat liver. The rat liver protein was cleaved with cyanogen bromide or trypsin and 17 of the resulting peptides were sequenced. DNA primers were generated from 3 peptides that had homology to sequences of the conserved catalytic domain of protein kinases. These primers were used in the polymerase chain reaction to obtain a 0.4-kilobase DNA fragment. This fragment was either radioactively labeled and hybridized to Northern blots of poly(A)$^+$ mRNA or used to screen a rat liver cDNA library. Northern blot analysis revealed four transcripts of 2.5, 3.2, 4.0, and 6.0 kilobases, and five S6 kinase clones were obtained by screening the library. Only two of the clones, which were identical, encoded a full-length protein. This protein had a molecular weight of 56,160, which correlated closely to that of the dephosphorylated kinase determined by SDS/PAGE. The catalytic domain of the kinase resembles that of other serine/threonine kinases belonging to the second messenger subfamily of protein kinases.**

An obligatory step in the mitogenic response is the activation and maintenance of high rates of protein synthesis throughout the G_1 phase of the cell cycle (1, 2). This increase is regulated at the level of initiation of translation and does not require *de novo* transcription (3, 4). This finding has led to the speculation that the activated growth factor receptor complex may mediate this response indirectly through the phosphorylation of key translational factors involved in initiation (5, 6). The most intensively studied of these proteins has been S6, which apparently resides in the mRNA binding site of the 40S ribosomal subunit (6). Within minutes of mitogen stimulation, S6 becomes multiply phosphorylated on five distinct serines located at the carboxyl end of the molecule (7). *In vitro* and *in vivo* studies argue that this event either triggers or, in concert with other initiation factors, facilitates the initiation of protein synthesis (8, 9).

Initial attempts to identify a mitogen-activated S6 kinase failed until phosphatase inhibitors such as β-glycerol phosphate were used during cell extraction (10, 11). This result led to the finding that the kinase was selectively inactivated by a type 2A phosphatase (12) and to its identification as a single polypeptide of M_r 70,000 (13). The kinase appears to phosphorylate the same sites in S6 as observed *in vivo* (13) and itself is activated by serine/threonine phosphorylation (14). More recently, the kinase was found to be biphasically activated, with the second phase of activation under the control of protein kinase C (15). Tryptic phosphopeptide maps of the enzyme from both phases are qualitatively similar, except for one unique phosphopeptide associated with the second phase of kinase activation (45). The results indicate that a single growth factor can activate two distinct signaling pathways, which converge on the same enzyme at different times (15).

A second mitogen-activated S6 kinase of M_r 92,000 has been detected in chicken embryo fibroblasts (16) with the use of antibodies against an S6 kinase purified from *Xenopus* eggs, termed S6 kinase II (17). Homologues to the *Xenopus* S6 kinase II cDNA (18) have also been cloned from mouse as well as chicken (19). Before that, this enzyme had only been detected in *Xenopus* eggs; in contrast, the M_r 70,000 S6 kinase has been purified from avian (20), mouse (13, 21), rat (22–24), rabbit (25), and bovine (26). The finding of S6 kinase II in chicken and mouse and its relatively larger molecular weight led to the suggestion that the M_r 70,000 kinase might be a proteolytic fragment of the M_r 92,000 S6 kinase II (19). To resolve this question, it was necessary to obtain either protein or cDNA sequence data for the M_r 70,000 kinase. The amount of protein recovered from fibroblasts was insufficient to initiate such studies (21). This problem was circumvented by purifying the kinase from the livers of rats that had first been injected with cycloheximide, an agent known to induce early mitogenic responses including S6 phosphorylation (22, 24).

Here we report on the use of protein sequence data to generate a 0.4-kilobase (kb) DNA fragment by the polymerase chain reaction (PCR) (27, 28). This fragment has been used for Northern blot analysis of poly(A)$^+$ mRNA and to search for corresponding cDNA clones in a rat liver cDNA library. One of the cDNA clones obtained has been sequenced[§] and shown to encode a serine/threonine kinase. The catalytic domain of this kinase has been compared to other known serine/threonine kinases.

MATERIALS AND METHODS

Peptide Sequencing. A more detailed protocol for this procedure will be reported elsewhere. In brief, ≈70 μg of purified S6 kinase was cleaved by either cyanogen bromide or trypsin and the resulting peptides were separated by SDS/PAGE or microbore HPLC (S.F. and G.T., unpublished data). The peptides were subjected to microsequencing using an Applied Biosystems 477A sequencer, equipped with a miniaturized reaction cartridge, by using rapid cycle chemistry and on-line analysis programs (N.T. and R. J. Mattaliano, unpublished data).

Nucleic Acids. RNA from rat embryo and liver was prepared by the method of Chomczynski and Sacchi (29). Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose. Oligo-

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Abbreviation: PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35864).

nucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. "Guess-mer" oligonucleotides based on codon usage frequencies have been designed from the peptide sequence presented in Fig. 1 (see refs. 30 and 31). Degenerate oligonucleotides used for PCR and DNA hybridization were purified by gel electrophoresis (32).

DNA Amplification and DNA Fragment Analysis. The PCR template was obtained by carrying out first-strand cDNA synthesis on rat embryo poly(A)$^+$ RNA (3 μg) using a Copykit (InVitrogen, San Diego, CA) with oligo(dT) primer. PCR was performed with a GeneAmp kit (Cetus) using 200 ng of rat embryo cDNA template and 100 pmol of degenerate oligonucleotide primers (Fig. 1). The amplification program was as follows: 1 min at 94°C, 2 min at 40°C, and 3 min at 72°C for 40 cycles followed by 15 min at 72°C. One microliter of the PCR product was reamplified for 30 cycles under the same conditions except the annealing temperature was raised from 40°C to 45°C. Both PCR products were electrophoresed in 2% agarose gels (BRL) and blotted on GeneScreen*Plus* (NEN). Guess-mer oligonucleotides (Fig. 1) were labeled by a fill-in reaction using 2.5 pmol of overlapping oligonucleotides; 100 pmol of dATP, dGTP, and dTTP; and 30 pmol of [α-^{32}P]dCTP with 1.6 units of Sequenase 2 polymerase (United States Biochemical) for 5 min at 37°C. The product was precipitated in the presence of tRNA and purified by gel electrophoresis as described above. Hybridization with the guess-mer probe and calf thymus DNA (200 μg/ml) was carried out at 42°C in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS and washed under the same conditions. Autoradiography revealed a 400-base-pair band that corresponded to a discrete fragment of the reamplified sample. This fragment was subcloned in Bluescript M13 KS− vector (Stratagene) for sequencing.

Probe Labeling by PCR. The labeling reaction was carried out following Schowalter and Sommer (34) in a vol of 20 μl containing 1 ng of template DNA; 1 μM degenerate primers (Fig. 1); 200 μM dCTP, dGTP, and dTTP; 10 μM dATP; and 2.5 μM [α-^{32}P]dATP (Amersham; >3000 Ci/mmol; 1 Ci = 37 GBq). The radiolabeled ATP and primers first had to be lyophilized to reach this concentration. The amplification program was the same as described above with annealing at 45°C. The labeled probe was separated from free nucleotides on a Sephadex G-50 spun column and analyzed on a 6% acrylamide gel (32). The specific activity of the product was 5.2 × 10^9 cpm/μg.

RNA Analysis. Rat liver poly(A)$^+$ RNA was electrophoresed in a 0.8% agarose gel containing 2.05 M formaldehyde, 20 mM Mops (pH 7), 0.5 mM EDTA, and 4 mM sodium acetate. The RNA was transferred to a nitrocellulose filter (Millipore) and prehybridized for 1 hr at 65°C in 10× SSC/10× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/0.1 M sodium phosphate, pH 6.2/2% glycine/calf thymus DNA (100 μg/ml). Hybridization was carried out for 16 hr at 65°C in 4 ml of 10× SSC/2× Denhardt's solution/40 mM sodium phosphate, pH 6.2/10% dextran sulfate/calf thymus DNA (100 μg/ml)/12 × 10^6 cpm of the PCR labeled probe per ml (5.2 × 10^9 cpm/μg). The filter was washed in 0.5× SSC/0.1% SDS at 65°C.

cDNA Library Screening. A rat liver cDNA library in Lambda ZAP II [Stratagene; 2 × 10^6 independent clones, oligo(dT) and random primed] was screened for S6 kinase clones. The phages were plated at 7 × 10^4 plaque-forming units per 135-mm plate and transferred in duplicate to nitrocellulose filters (Millipore). Prehybridization was carried out for 1 hr at 65°C in 2.5 ml of 5× SSC per filter/10× Denhardt's solution/0.1% SDS/calf thymus DNA (250 μg/ml). Hybridization was for 16 hr under the same conditions with 6 × 10^6 cpm of PCR labeled probe per filter. The filters were washed with 0.5× SSC/0.1% SDS at 65°C. Positive clones found in duplicate were submitted to a secondary screening. *In vivo* excision of Bluescript plasmid was carried out from the Lambda ZAP II vector by coinfecting with helper virus (Stratagene). The clones in Bluescript SK− were used for DNA sequencing.

DNA Sequencing. Nucleic acid sequence determination was performed by the dideoxynucleotide chain-termination method (35) using the Sequenase 2 sequencing kit (United States Biochemical). Double-strand sequencing (36) was carried out on Bluescript plasmids with the vector primers (Stratagene) and specific oligonucleotide priming (37). The University of Wisconsin Genetics Computer Group programs were used to search for sequence homologies

RESULTS

Generation of cDNA Probe. The strategy used in generating a specific cDNA probe to the S6 kinase is outlined in Fig. 1. Cycloheximide-activated S6 kinase purified from rat liver was cleaved with either cyanogen bromide or trypsin and the resulting peptides were resolved by SDS/PAGE or microbore reverse-phase HPLC, respectively. Under these conditions, a number of peptides were generated, 17 of which were sequenced. Three of the initial peptides sequenced had evident homology with subdomains VI, IX, and XI of the conserved catalytic domain of serine/threonine kinases (ref. 33; Fig. 1). On the basis of this observation, degenerate oligonucleotides representing the sequence in domains VI to XI were synthesized and a 0.4-kb DNA fragment was generated by PCR with rat embryo cDNA used as template (Fig. 1). The size of the DNA fragment roughly fit with that predicted for a peptide extending from subdomain VI to XI (33) and hybridized on Southern blots with a guess-mer derived from the peptide presumed to represent S6 kinase subdomain IX. Subcloning and sequencing of the 0.4-kb fragment revealed that it encoded the S6 kinase peptide fragment presumed to represent subdomain IX. On the basis of its partial DNA sequence it was judged that this DNA probe would hybridize specifically to the S6 kinase mRNA and cDNA.

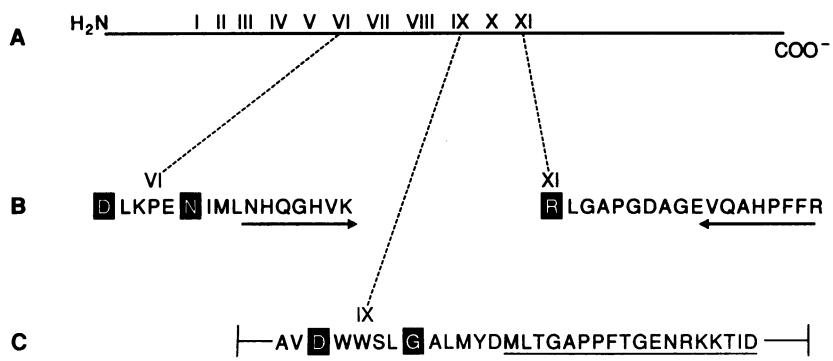


FIG. 1. Strategy for cloning S6 kinase. (*A*) Hypothesized S6 kinase showing conserved regions of the catalytic domain (33). (*B*) Protein sequences of peptides suspected of residing in subdomains VI and XI. Amino acids are indicated by single-letter code. Solid boxes represent invariant residues in protein kinases (33). Arrows indicate sequences used to generate sense (→) or antisense (←) degenerate oligonucleotides used in the PCR. (*C*) Protein sequence of peptide in subdomain IX. Solid boxes are the same as in *B*. Underlining indicates sequence used to generate a guess-mer (30, 31).

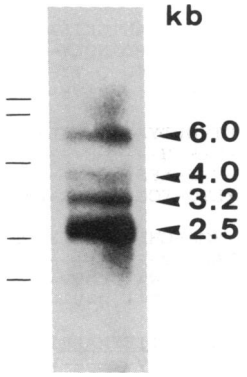
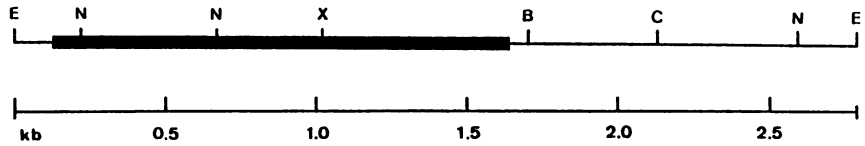


FIG. 2. Northern blot analysis of rat liver mRNA. The 0.4-kb DNA fragment was radioactively labeled and hybridized to a Northern blot containing 3 μg of poly(A)$^+$ mRNA. Lines on the left indicate RNA size markers (BRL) and are (from top to bottom) 9.5, 7.5, 4.4, 2.4, and 1.4 kb, respectively. Sizes on the right indicate mRNA transcripts that hybridized to the radioactive 0.4-kb DNA fragment.

Northern Blot Analysis. The 0.4-kb DNA fragment representing subdomains VI–XI was used to probe a Northern blot of rat liver poly(A)$^+$ mRNA to determine the diversity and size of the S6 kinase mRNA transcripts. It was assumed from the apparent low abundance of the S6 kinase protein that the mRNA would be rare, and so a probe of high specific activity was required for Northern blot analysis and for screening cDNA libraries. We therefore turned to a recent method using PCR to generate DNA probes of <0.5 kb having specific activities of 0.5–1.0 × 10^{10} cpm/μg (34). The 0.4-kb S6 kinase fragment hybridized by Northern blot analysis to four transcripts of 2.5, 3.2, 4.0, and 6.0 kb (Fig. 2).

cDNA Clones Encoding S6 Kinase. To isolate cDNA clones encoding the S6 kinase, a Lambda ZAP II rat liver cDNA library was screened with the radioactively labeled 0.4-kb DNA fragment. From 1.6 × 10^6 clones, 5 positive clones were obtained, designated 4, 6, 7, 15, and 17 with insert sizes of 2.8, 1.5, 2.0, 2.8, and 1.4 kb, respectively. Each clone was partially sequenced by using an internal primer derived from the 0.4-kb fragment, and each was found to contain the S6 kinase sequence. More extensive sequencing revealed that clones 6, 7, and 17 were incomplete in the 5′ coding region, and they were not considered further. By restriction map analysis (Fig. 3*a*) and partial sequencing, clones 4 and 15 appeared to be identical, reflecting the fact that the library had been amplified one time. The insert in clone 4 was sequenced and found to contain 2806 nucleotides. By aligning the peptide sequences with the DNA sequence (Fig. 3*b*), the third ATG from the 5′ end of the clone has been assigned the translation start site (nucleotide +1). The preceding ATGs in this frame (−111 and −48) are followed by a stop codon at −27. The presumed ATG translation initiation codon has a G in positions −3 and +4, indicating that this is a strong start site for translation (38). The 5′ untranslated region is 133 nucleotides long, consistent with the average length found in most mRNAs sequenced to date (38). The sequence encoding the S6 kinase gene contains 1506 nucleotides, representing a protein of 502 amino acids, and is terminated by two consecutive translation stop codons. Sequencing from the 3′ end

a



b

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TCCTATAACCCACCTCAGGGTTATGGACAACAGAACCAGTACAACAGCAGCAGTGGAGGTGGTGGAGGGGGCGGTGGAGGCAACTATGGCCAAGATCAGTCCTCTATGAGTGGCGGCGGC  120
GGTGGTGGTGGTTATGGCAGGAGTGTTTGACATAGACCTGGACCAGCCAGAGGATGCAGGCTCTGAGGATGAGCTGGAGGAGGGGGGTCAGTTAAATGAAAGCATGGACCATGGGGGAGT  240
          M  A  G  V  F  D  I  D  L  D  Q  P  E  D  A  G  S  E  D  E  L  E  E  G  G  Q  L  N  E  S  M  D  H  G  G  V

TGGACCATATGAACTTGGCATGGAACATTGTGAGAAATTTGAAATCTCAGAAACTAGTGTGAACAGAGGGCCAGAAAAAATCAGACCAGAATGTTTTGAGCTACTTCGGGTACTTGGTAA  360
  G  P  Y  E  L  G  M  E  H  C  E  K  F  E  I  S  E  T  S  V  N  R  G  P  E  K  I  R  P  E  C  F  E  L  L  R  V  L  G  K

AGGGGGCTATGGAAAGGTTTTTCAAGTACGAAAAGTAACAGGAGCAAATACTGGGAAGATATTTGCCATGAAGGTGCTTAAAAAGGCAATGATAGTAAGAAATGCTAAAGATACAGCTCA  480
  G  G  Y  G  K  V  F  Q  V  R  K  V  T  G  A  N  T  G  K  I  F  A  M  K  V  L  K  K  A  M  I  V  R  N  A  K  D  T  A  H

TACAAAAGCAGAGCGGAATATTCTGGAGGAAGTAAAGCATCCCTTCATCGTGGATTTAATTTATGCCTTTCAGACCGGTGGAAAACTCTACCTCATCCTTGAGTATCTCAGTGGAGGAGA  600
  T  K  A  E  R  N  I  L  E  E  V  K  H  P  F  I  V  D  L  I  Y  A  F  Q  T  G  G  K  L  Y  L  I  L  E  Y  L  S  G  G  E

ACTATTTATGCAGTTAGAAAGAGAGGGGATATTCATGGAAGATACAGCTTGCTTTTACTTGGCTGAAATCTCCATGGCTTTGGGGCATTTACATCAAAAAGGGATCATCTACAGAGACCT  720
  L  F  M  Q  L  E  R  E  G  I  F  M  E  D  T  A  C  F  Y  L  A  E  I  S  M  A  L  G  H  L  H  Q  K  G  I  I  Y  R  D  L

GAAGCCGGAGAACATCATGCTTAATCACCAAGGTCACGTGAAGCTGACAGACTTTGGACTATGCAAAGAATCTATTCATGATGGAACAGTCACGCACACATTTTGTGGAACAATAGAATA  840
  K  P  E  N  I  M  L  N  H  Q  G  H  V  K  L  T  D  F  G  L  C  K  E  S  I  H  D  G  T  V  T  H  T  F  C  G  T  I  E  Y

CATGGCCCCTGAAATCTTGATGAGAAGCGGCCACAACCGTGCTGTGGATTGGTGGAGTTTGGGAGCATTAATGTATGACATGCTGACTGGAGCACCTCCATTCACTGGGGAGAATAGAAA  960
  M  A  P  E  I  L  M  R  S  G  H  N  R  A  V  D  W  W  S  L  G  A  L  M  Y  D  M  L  T  G  A  P  P  F  T  G  E  N  R  K

GAAAACAATTGACAAAATCCTCAAATGTAAACTTAATTTGCCTCCCTACCTCACACAAGAAGCTCGAGATCTGCTTAAAAAGCTGCTGAAAAGAAATGCTGCTTCTCGTCTTGGAGCTGG 1080
  K  T  I  D  K  I  L  K  C  K  L  N  L  P  P  Y  L  T  Q  E  A  R  D  L  L  K  K  L  L  K  R  N  A  A  S  R  L  G  A  G

CCCTGGGGATGCTGGAGAAGTCCAAGCGCATCCATTTTTTAGACACATTAACTGGGAAGAGCTTTTGGCTCGGAAGGTGGAGCCCCCCTTTAAGCCTCTGTTGCAATCTGAAGAGGATGT 1200
  P  G  D  A  G  E  V  Q  A  H  P  F  F  R  H  I  N  W  E  E  L  L  A  R  K  V  E  P  P  F  K  P  L  L  Q  S  E  E  D  V

GAGTCAGTTTGATTCAAAGTTTACTCGTCAGACACCTGTTGACAGCCCCGATGACTCAACTCTCAGTGAAAGTGCCAACCAGGTCTTTCTGGGTTTTACATATGTGGCTCCATCTGTACT 1320
  S  Q  F  D  S  K  F  T  R  Q  T  P  V  D  S  P  D  D  S  T  L  S  E  S  A  N  Q  V  F  L  G  F  T  Y  V  A  P  S  V  L

TGAAAGTGTGAAAGAAAAGTTTTCTTTTGAACCAAAAATCCGATCGCCTCGAAGATTTATTGGTAGCCCACGAACGCCTGTCAGCCCAGTCAAATTCTCTCCTGGGGATTTCTGGGGACG 1440
  E  S  V  K  E  K  F  S  F  E  P  K  I  R  S  P  R  R  F  I  G  S  P  R  T  P  V  S  P  V  K  F  S  P  G  D  F  W  G  R

AGGTGCTTCAGCCAGCACAGCAAATCCTCAGACACCTGTGGAATACCCAATGGAAACAAGTGGAATAGAGCAGATGGATGTGACAACGAGCGGGGAAGCTTCAGCGCCACTTCCAATCCG 1560
  G  A  S  A  S  T  A  N  P  Q  T  P  V  E  Y  P  M  E  T  S  G  I  E  Q  M  D  V  T  T  S  G  E  A  S  A  P  L  P  I  R

ACAGCCCAACTCTGGGCCATACAAAAAACAAGCTTTTCCTATGATCTCCAAACGGCCAGAGCACCTGCGTATGAATCTATGATGAAACAATGCTTTTATTAATGCAAATGCAAAAAGGAA 1680
  Q  P  N  S  G  P  Y  K  K  Q  A  F  P  M  I  S  K  R  P  E  H  L  R  M  N  L

ACAAATCCTGGAAGGGGATGTGTGAGGATCCTGCAAGATAAAAATGAGAAAATGGCAGTCTCAAAGAGTCAGTGTCATTACCTGGAATGCTTTCGATGGAGGAAAAAATAAACATGGATT 1800
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FIG. 3. Restriction endonuclease map of clones 4 and 15 and cDNA and amino acid sequences of S6 kinase. (*a*) Heavy line indicates coding region. B, *Bam*HI; C, *Cla* I; E, *Eco*RI; N, *Nco* I, X, *Xho* I. (*b*) Nucleotides and amino acids are denoted by single-letter code. The coding region contains 502 amino acids. Stars indicate start and stop codons. Arrows indicate nucleotides in positions −3 and +4. Upper and lower underlinings denote tryptic or cyanogen bromide peptide sequences, respectively.

of the gene did not reveal a poly(A) tail, suggesting that the cDNA clone had been initiated by random priming. Of the 17 peptides sequenced, all but 1 could be aligned with the cDNA sequence (Fig. 3*b*).

SDS/PAGE of Dephosphorylated S6 Kinase. The predicted molecular weight of the protein encoded by clone 4 (M_r 56,160) is substantially smaller than the M_r of 70,000 that we reported previously for the purified S6 kinase (13). In these studies, the kinase was subjected to electrophoresis on 15% acrylamide gels containing low concentrations (0.09%) of bisacrylamide (32). However, we reported earlier (13) that the protein migrates faster on 10% acrylamide gels containing the usual concentration (0.27%) of bisacrylamide (39). In addition, removal of phosphate groups causes a substantial reduction in its molecular weight (14). Thus, the molecular weight of the kinase may be closer to the value obtained from the cDNA sequence. To test this possibility, an aliquot of purified S6 kinase was incubated in the absence and presence of the purified catalytic subunit of phosphatase 2A. Under the conditions used, phosphatase 2A rendered the enzyme totally inactive toward itself and toward S6 and should have removed all phosphate groups (14). After this treatment, the mobility of both kinase preparations was measured on 10% acrylamide gels containing the usual concentrations of bisacrylamide. The results show that the activated kinase migrates at M_r 63,000, but the dephosphorylated enzyme migrates substantially faster at M_r 60,000 (Fig. 4). Thus, the apparent anomaly in molecular weights may in part be explained by the gel system used and by posttranslational modifications.

Comparison of Catalytic Domains. In examining the protein sequences contained within the catalytic domain of the S6 kinase, all of the nine invariant amino acids and the additional five found in all but 1 of the 65 protein sequences analyzed (33) are also present in this enzyme (Fig. 5). Finally, the motifs DLKPEN and G(T/S)XX(Y/F)XAPE in subdomains VI and VIII identify the S6 kinase as a member of the serine/threonine kinase family (Fig. 5). Of the five major subfamilies defined by amino acid sequence comparison, the S6 kinase appears to fall into the protein kinase C subfamily. The most striking difference with this subfamily is that the S6 kinase would be the only member to contain tyrosine rather than phenylalanine at position 79 (Fig. 5). Within this subfamily, the S6 kinase is most closely related to the first catalytic domain of S6 kinase IIα (18) from *Xenopus* (56%), yeast protein kinase 1 (40) from *Saccharomyces cerevisiae* (49%), and protein kinase C_ε (41) (44%). However, these identities fall off sharply outside of the catalytic domain, with yeast protein kinase 1 having the highest overall homology of 41%. Together the results show that the S6 kinase is a member of the serine/threonine family of protein kinases, most closely related to the group of kinases activated by second messengers.

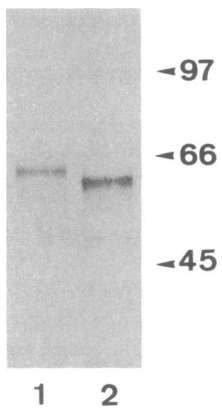

FIG. 4. Silver-stained SDS/polyacrylamide gel of S6 kinase. S6 kinase (50 ng) was incubated in the absence (lane 1) or presence (lane 2) of 1 unit of phosphatase 2A for 1 hr at 37°C. Relative molecular weights ($\times 10^{-3}$) of BRL protein standards are indicated on the right: phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), and ovalbumin (M_r 45,000).

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  S6K  (66) ECFELLRVLGKGGYGKVFQVRKVTGANTGKIFAMKV
 S6KII  (60) SD-V--K---Q-SF----L---I-PPDANQLY----
  YPK1 (345) DD-D--K-I---SF---M-------..KD-Q-VY-L-A
  PKCε (406) DE-NFIK-----SF---M.LAELK-..KDEVY-V--

LKKAMIVRNAKDTAHTKAERNILE.EVKHPFIVDLIYAFQTGGKLYLI
----TL..KVR-RVR--M--D--A.D-H---V-R-H-----E------
IR-SY--SKS.EVT--L---TV-A.R-DC----P-KFS--SPE---FV
---DV-LQD.D-VDC-MT-KR--ALAR---YLTQ-YCC---KDR-FFV

LEYLSGGELFMQLEREGIFMEDTACFYLAEISMALGHLHQKGIIYRDL
-DF-R--D--TR-SK-VM-T-EDVK-----LALG-D---SL-------
-AFIN-----YH-QK--R-DLSR-R--T--LLC--DN--KLDVV----
M--VN--D-MF-IQ-SRK-D-PRSG--A--VTS--MF---H-V-----

KPENIMLNHQGHVKLTDFGLCKESIHDGTVTHTFCGTIEYMAPEILMR
-----L-DEE--I-------S--A-DHEKKAYS----V------VVN-
-----L-DY---IA-C------LNMK-DDK-D-----P--L---L-LG
-LD--L-DAE--S--A---M---G-LN-VT-T-----PD-I-----QE

SGHNRAVDWWSLGALMYDMLTGAPPFTGENRKKTIDKILKCKLNLPPY
Q--SHSA----Y-V--FE----SL--Q-KD--E-MTL---A--GM-QF
L-YTK-----T--V-L-E----L--YYD-DVP-IYK---QEP-VF-DG
LEYGPS----A--V---E-MA-Q---EAD-EDDLFES--HDDVLY-VW

LTQEARDLLKKLLKRNAASRLGAGPGDAGE..VQAHPFFRHI (332)
-SN--QS--RA-F---PTN---SAMEG-E-..IKRQ---ST- (324)
FDRD-K---IG--S-DPTR---Y..NG-D-..IRN----SQL (605)
-SK--VSI--AFMTK-PHK---CVAAQN--DAIKQ----KE- (671)
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FIG. 5. Comparison of S6 kinase catalytic domain with other known protein kinases. S6K, rat liver S6 kinase; S6KII, *Xenopus laevis* S6 kinase II$_\alpha$; YPK1, yeast protein kinase 1; PKCε, rat protein kinase C$_\varepsilon$. Dashes indicate identity with amino acids in the S6K gene. Stars indicate invariable amino acids or those in all but one of the serine/threonine kinases examined (33). Numbers indicate beginning and end of catalytic domains.

DISCUSSION

Earlier lines of evidence supported the view that the purified M_r 70,000 protein kinase present in Swiss mouse 3T3 cells and rat liver was the S6 kinase. This evidence included cochromatography of S6 kinase activity with the purified M_r 70,000 protein, apparent ability to autophosphorylate, and a high specific activity toward S6 (21, 22, 24). This conclusion was strongly supported by our initial protein sequence data showing that a number of the peptides derived from this protein contain highly conserved amino acid sequence motifs specific for protein kinases and one unique to serine/threonine kinases (Fig. 1). This result was confirmed by sequencing a cDNA clone containing the entire coding region of the kinase (Fig. 4). However, the fact that the gene sequence represented a protein of substantially lower molecular weight than previously predicted by SDS/PAGE (13), and that one of the peptides could not be accounted for initially, put this view into question. As shown in Fig. 4, the apparent difference between the molecular weight determined by gel electrophoresis (13) and that predicted by the cDNA may be explained by the SDS/PAGE system used and the phosphorylation state of the protein. This question should be resolved by comparing the electrophoretic mobilities on SDS/PAGE of the dephosphorylated kinase and the *in vitro* translation product of clone 4. The additional peptide may be attributable to a contaminating protein or to a second gene product (see below). We have noted a protein of almost the identical molecular weight that cochromatographs with the S6 kinase up to the last step of purification. By a number of criteria this protein seems closely related to the S6 kinase, although it contains a different N-terminal sequence (S.F., H. A. Lane, and J. Hofsteenge, unpublished data). Further sequencing studies should resolve whether these two proteins are related.

The N-terminal sequence of the S6 kinase could not be determined because it was blocked. However, the cDNA contains a stop signal upstream of the proposed "strong" ATG start site (38). Besides containing G in the critical −3

and +4 positions of the translation initiation site, G is also found in the first position of each of the eight nucleotide triplet sequences preceding the putative initiator ATG. Such a repetition is observed in positions −3, −6, and −9 in a large number of mRNA sequences (38). It has been argued that this motif allows ribosomes to carry out "frame monitoring" upstream of the initiator codon (38). It should also be noted that the S6 kinase gene is unusual in having T rather than C in positions −1 and −2. This arrangement has no measurable effect on initiation if a purine is present at −3 and if there is a G in position +4 (38). From the Northern blot analysis (Fig. 2), it appears that the 2.5-kb transcript is the most highly represented mRNA in rat liver poly(A)$^+$ RNA. However, because clone 4 appears to encode a longer mRNA and the 3′ untranslated region is incomplete, it may be derived from one of the larger mRNA transcripts. It should be noted that all the transcripts appear to be S6 kinase specific because a PCR fragment from the nonconserved region (nucleotides 164–343 from the 5′ end) revealed the identical transcripts shown in Fig. 2 (unpublished data). These multiple transcripts may be explained by preliminary results indicating the existence of a gene family as well as differential mRNA processing (S.C.K., P.B., and G.T., unpublished data).

It has been suggested that the M_r 70,000 S6 kinase may represent a proteolytic cleavage product of the M_r 92,000 S6 kinase II, even though antibodies against the M_r 92,000 kinase do not appear to immunoprecipitate the M_r 70,000 S6 kinase (17). It is clear from the comparison of protein and cDNA sequence data that this is not the case (Fig. 5). Furthermore, of the two kinase catalytic domains in S6 kinase II, the first, or protein kinase C-like, appears to be the domain that is active toward S6, due to its similarity to the M_r 70,000 S6 kinase. Five S6 kinase II cDNAs have been cloned—two from *Xenopus* (18), one from chicken, and two from mouse (19). In comparing the S6 kinase catalytic domain to that of the S6 kinase II sequences, the highest homology (58%) was with the mouse partial clone, designated rsk^{mo}-2. However, none of these S6 kinase II sequences shows homology to the S6 kinase outside of the catalytic domain except for a short stretch of amino acids following subdomain XI (positions 333–372 of the S6 kinase). Thus, even though these two proteins are related through their kinase domains, they are clearly distinct enzymes. This raises the question of why a cell would require two kinases to phosphorylate S6. There are several possible explanations including (*i*) different mitogens acting through only one of the two kinases, (*ii*) each kinase phosphorylates distinct sites on S6, or (*iii*) in the intact cell only one kinase is responsible for modulating S6 phosphorylation.

The S6 kinase appears to belong to the subfamily of kinases regulated by second messengers such as cAMP or phospholipids (33). However, as pointed out earlier, the S6 kinase has a tyrosine in position 79 rather than a phenylalanine, as is found in the other members of this subfamily (33). This tyrosine is found in all of the members of the cdc-28/cdc-2 subfamily and in each case it is phosphorylated (42). Removal of this phosphate leads to activation of the cdc-28/cdc-2 kinase (42) and premature entry into mitosis. Furthermore, unlike the kinases of the second messenger subfamily, S6 kinase appears to be activated by phosphorylation (14). The same is true of S6 kinase II (43) and the microtubule-associated protein 2 kinase (44). When more kinases that are activated in this manner have been cloned and sequenced, it may be possible to determine whether they represent a unique family.

Note in Proof. Since submission of this manuscript for publication, we learned that a closely related S6 kinase cDNA had been cloned and sequenced (46). Comparison of the sequence reported here to this clone revealed that they differ at their 5′ ends and that the peptide missing from our cDNA sequence is also absent from their sequence.

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