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A single gene encodes two isoforms of the p70 S6 kinase: Activation upon mitogenic stimulation

(gene and mRNA analyses/immunoprecipitation/serum-stimulated 3T3 cells)

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ABSTRACT **Previously, two cDNA clones were isolated from a rat liver or hepatoma cDNA library for the mitogenic-activated p70 S6 kinase ($p70^{s6k}$). Except for a single amino acid change and a 23-amino acid N-terminal extension in the latter clone, the open reading frames of the two clones are identical. A probe common to both clones also revealed four distinct transcripts. Here, by using specific probes, it was possible to show which transcript corresponds to which clone and that both clones are derived from the same gene. Furthermore, analysis of *in vitro* translation products using specific antibodies demonstrates that both clones encode the $p70^{s6k}$ but that the clone harboring the 23-amino acid extension also encodes an additional isoform of the kinase, referred to as $p85^{s6k}$. It could be shown by using the specific antibody to the $p85^{s6k}$ that this isoform of the kinase is present in rat liver and is activated after mitogenic stimulation of quiescent Swiss 3T3 cells.**

Phosphorylation of 40S ribosomal protein S6 is an apparent prerequisite for activation of protein synthesis and cell growth in response to numerous growth factors and oncogene products (see refs. 1 and 2). This event is largely regulated by the mitogen-activated M_r 70,000 S6 kinase (3–5) or $p70^{s6k}$. The kinase is biphasically activated by serine/threonine phosphorylation through at least two distinct signaling pathways (5–7) is highly specific for S6, and has a K_m of 0.25 μM for the substrate (8). It is able to phosphorylate four of the five sites observed *in vivo* (9, 10), with recognition of these sites dependent on a block of three arginines, $S6_{231-233}$ (8). The kinase responsible for activating $p70^{s6k}$ has not yet been identified, but unlike the p90 family of S6 kinases (1, 2), a distinct kinase family with wide substrate specificity, it does not appear to be the p42 mitogen-activated protein (MAP) kinase (11, 12).

Recently, protein sequence data for $p70^{s6k}$ was obtained from the livers of rats injected with cycloheximide (13–15), an agent known to turn on many of the early mitogenic responses (16, 17). The sequence information was used to isolate two distinct cDNA clones, one from a rat liver cDNA library and one from a rat hepatoma cDNA library (18, 19), referred to here as clones 1 and 2, respectively (Fig. 1). Clone 1 was 2.8 kilobases (kb) and contained a 133-nucleotide (nt) G+C-rich, 5′ untranslated region followed by a 502-amino acid residue coding sequence. The nucleotide sequence surrounding the initiation codon conformed to a "strong" consensus translation start site (18, 20). The 3′ untranslated region was A+T-rich and devoid of a poly(A) tail. Clone 2 was 2.3 kb and contained a short (21 nt) 5′ untranslated region followed by a "weak" consensus translation start site and an open reading frame capable of encoding a 525-amino acid protein. A cDNA clone almost identical to clone 2 has also been isolated from a rabbit cDNA library (21). A second AUG start codon in clone 2 has been identified at nt 91 (19), which corresponds to the strong translation start site of clone 1 (18). From this second start site, the coding region of clone 2 was identical to that of clone 1 except for a single amino acid (Pro-344 in clone 1 and Arg-367 in clone 2). The 3′ untranslated region of clone 2 was shorter, but identical to clone 1, and ended in a poly(A) tail from nt 2283 (19). The predicted molecular weights of clones 1 and 2 were 56,200 and 59,200, respectively, very close to the molecular weight observed for the dephosphorylated $p70^{s6k}$ by Laemmli SDS/PAGE (18, 22). In addition, a DNA probe common to both clones revealed four S6 kinase transcripts (18). These observations raised questions concerning whether both clones were derived from the same or different genes, which clone encoded the $p70^{s6k}$, and whether a second isoform of the kinase exists.

Here we have used specific probes to determine the transcripts to which clones 1 and 2 correspond as well as the number of $p70^{s6k}$ genes. Next, an anti-peptide antibody that recognizes the translation products of clones 1 and 2, or one that recognized only that of clone 2, was used to determine which of the two clones encoded the $p70^{s6k}$. Finally, these antibodies were used to determine whether a second form of the kinase was present *in vivo* and whether this form was activated after mitogenic stimulation of quiescent cells.

MATERIALS AND METHODS

Plasmid Constructs. Clone 1 s6k cDNA was obtained either from the original 2.8-kb clone (18) or from a shorter 1.6-kb fragment constructed as follows: an *Xba* I site was introduced into the original 2.8-kb clone at nt 122 by using synthetic oligonucleotides and the PCR (18). The DNA fragment contained between this new *Xba* I site and the *Bam*HI site (nt 1706) has been subcloned in pBluescript KS− and is referred to as p2B4. Clone 2 s6k cDNA was generated as follows: a DNA fragment containing clone 2 (19) was first amplified by PCR from a rat liver cDNA library (Stratagene) by using oligonucleotide primers based on the published sequence (19). After two rounds of amplification with nested primers, a single 411-nt fragment was obtained. This fragment included a *Not* I site at the 5′ end of the sense primer and a *Spe* I site at nt 256 (19). The 171-nt *Not* I/*Spe* I restriction fragment from p2B4 was replaced by the 239-nt *Not* I/*Spe* I DNA fragment specific for clone 2 to create plasmid p2BK.

DNA Probe Labeling. DNA probes >250 nt were radioactively labeled by random priming with the Prime-It kit (Stratagene). Shorter probes were labeled by PCR as described (23). Specific activities of the random-primed and PCR probes were $\approx 2 \times 10^8$ cpm per μg of DNA and 5×10^9 cpm per μg of DNA, respectively.

Northern Blot and Southern Blot Analysis. For Northern blot analysis, rat liver poly(A)$^+$ RNA was isolated and separated as described (18, 24) but was passed three times

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Abbreviation: nt, nucleotide(s).

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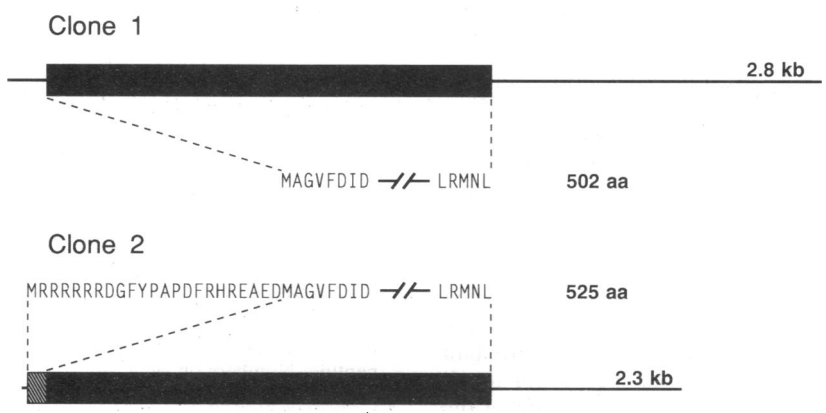

FIG. 1. Rat s6k cDNA clones 1 and 2. Solid thick bar indicates common coding region; shaded thick bar indicates 5′ coding region specific for clone 2; thin bar indicates untranslated regions. Amino acids (aa) are denoted by single-letter code.

over an oligo(dT) column. After transfer to a nylon membrane (Hybond N; Amersham) (25), prehybridization was carried out for 1 hr at 68°C in 2× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) containing 5× Denhardt's solution, 1% (wt/vol) SDS, and 100 μg of calf thymus DNA per ml (25). Hybridization with a radiolabeled DNA probe was accomplished by overnight incubation at 68°C in the same buffer supplemented with 10% (wt/vol) dextran sulfate. High-stringency washing was performed by decreasing salt to a final concentration of 0.1× SSPE in 0.1% SDS for 20 min at 68°C. Low-stringency washing was done in a solution of 0.2× SSPE in 0.1% SDS for 10 min at 65°C. For Southern blot analysis, genomic DNA was isolated from rat liver nuclei (25) and 10-μg aliquots were digested as described in the text and were separated overnight on 0.7% agarose gels in 0.5× TBE (25). Transfer to Hybond N membranes was carried out according to the manufacturer's instructions. Hybridization and washing of the blots were carried out as described above.

***In Vitro* Translation of s6k cDNA Clones.** The p2B4 and p2BK plasmids were linearized with *Bam*HI and transcribed *in vitro* with the T7 promotor contained in the pBluescript KS− vector. The transcripts were translated in an mRNA-dependent rabbit reticulocyte lysate (26) for 1 hr at 30°C in the presence of 3.6 μCi of [^{35}S]methionine (Amersham) per 10-μl reaction mixture (1 Ci = 37 GBq).

Anti-Peptide Antibodies. Rabbit polyclonal anti-peptide antibodies were generated by using synthetic peptides representing amino acids 1–31 of clone 1 (18), referred to as p70^{s6k} M5 antiserum (27), and amino acids 8–23 of clone 2 (19). Coupling of peptides and immunization of the rabbits were carried out essentially as described (28).

Immunoblotting, Immunoprecipitation, and S6 Kinase Assay. After SDS/PAGE (22), proteins were electrophoretically transferred to Immobilon membranes (Millipore) and stained with 2% (wt/vol) Ponceau red in 3% (vol/vol) chloric acid to visualize the molecular weight markers. After blocking with 5% skim milk (Fluka), the blot was incubated overnight with the appropriate antiserum at 4°C with gentle agitation. Bound antibody was visualized with a porcine anti-rabbit antibody conjugated to alkaline phosphatase (28). Extracts for immunoprecipitations or S6 kinase assays were prepared from Swiss mouse 3T3 cells in lysis buffer (120 mM NaCl/20 mM NaF/1 mM benzamidine/1 mM EDTA/6 mM EGTA/15 mM PP_i/1% Nonidet P-40/30 mM 4-nitrophenylphosphate/0.5 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/50 mM Tris·HCl, pH 8.0) on ice and then homogenized with a Polytron (Kinematica, Lucerne, Switzerland) three times at full speed for 3 sec. For immunoprecipitation, cell extract samples (50–200 μg) or rabbit reticulocyte translation products (6–10 μl) were diluted in lysis buffer to a final vol of 200 μl and then 5 μl of the indicated antiserum was added. After incubation of the mixture on ice for 2 hr, 20 μl of 50% (wet vol/vol) protein A-Sepharose was added and the mixture was incubated for an additional 30 min. The protein A immune complex was collected by centrifugation, washed four times in lysis buffer, washed one time with dilution buffer (5) and, finally, resuspended in 5 μl of dilution buffer containing 1 mM dithiothreitol. The suspended protein A complexes or whole-cell extracts were assayed for S6 kinase activity (5).

RESULTS

Northern Blot Analysis. To determine whether either clone 1 or clone 2 could be assigned to specific mRNA transcripts, Northern blots of rat liver poly(A)$^+$ RNA were hybridized with DNA probes representing sequences common or unique to both clones (Fig. 1). A probe comprising the entire coding region of clone 1, and most of clone 2, revealed four transcripts of 6.0, 4.0, 3.2, and 2.5 kb (Fig. 2, lane 1). However, a probe representing only the 5′ untranslated region of clone 1 hybridized to the 6.0-, 4.0-, and 3.2-kb transcripts and not to the 2.5-kb transcript (lanes 2 and 3), even after less stringent washing of the membrane (lane 3). This probe also bound to an abundant 2.0-kb transcript, which was clearly distinguishable from the p70^{s6k} transcripts (compare lane 1 with lanes 2 and 3). A probe unique to the 3′ untranslated region of clone 1 also bound to the 6.0-, 4.0-, and 3.2-kb transcripts (lane 4) but not to the 2.5-kb transcript. In contrast to the clone 1-specific probes, a probe derived from the 5′ end of clone 2 hybridizes strongly to the 2.5-kb transcript as well as to the 6.0-kb transcript, but it did not bind to either the 4.0-

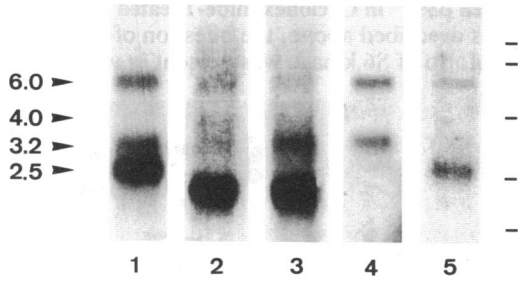

FIG. 2. Northern blot analyses of rat liver mRNA. Poly(A)$^+$ RNA (3 μg per lane) was hybridized with specific DNA probes and washed under high-stringency conditions unless otherwise stated. The following probes were used in each lane: 1, common coding region (nt 122–1706) (18); 2 and 3, 5′ end of clone 1 (nt 1–90); 3, low-stringency wash; 4, 3′ end of clone 1 (nt 2333–2806); 5, 5′ end of clone 2 (nt 14–97). Sizes of transcripts are indicated on left and lines on right side show RNA size markers: 9.5, 7.5, 4.4, 2.4, and 1.4 kb (BRL).

or the 3.2-kb transcript (lane 5). The results indicated that the 6.0-kb transcript contained sequences specific for both clones, that the 4.0- and 3.2-kb transcripts correspond to clone 1 and the 2.5-kb transcript corresponds to clone 2.

Southern Blot Analysis. Considering the results described above and the fact that clones 1 and 2 share a common 3′ untranslated region, the two clones may be derived from a single gene. In addition, preliminary genomic PCR analyses, using as primers a sequence specific for the 5′ coding region of clone 2 and a sequence from the translation start site of clone 1, indicated that these two regions were contiguous in the gene (data not shown). It was reasoned that if the *s6k* gene was a single gene, then identical genomic Southern blot patterns should be obtained with probes specific for the 5′ coding region of clone 2 and a short probe beginning at the AUG start site of clone 1. A probe spanning the entire coding region of clone 1 revealed a broad range of bands (Fig. 3*A*), indicative of either several genes or a single large gene, whereas a DNA probe specific for the 5′ end of the clone 2 coding sequence only hybridized to a subset of these bands, regardless of the restriction enzyme used (Fig. 3*B*). The same pattern was obtained when a probe specific for the beginning of the clone 1 coding region was used (Fig. 3*C*), as would be expected if a single copy of the gene existed. The results demonstrate that *s6k* is a single gene and show that there were no full-length *s6k* pseudogenes present in the rat genome.

Expression of Clones 1 and 2. To determine which clone was responsible for encoding the $p70^{s6k}$, each was translated *in vitro* and their products were immunoprecipitated with a specific anti-peptide antibody generated against the N terminus of clone 1. As shown in Fig. 4 (lane 1), a single band of M_r 65,000, identical in size to that obtained by SDS/PAGE (22) for the dephosphorylated kinase (data not shown), was immunoprecipitated from the translation products of clone 1. In contrast, two bands of M_r 65,000 and 85,000 (lane 3) are precipitated from the translation products of clone 2. In both cases, immunoprecipitation was blocked by competing peptide (lanes 2 and 4). The results suggested that both clones are capable of expressing the $p70^{s6k}$ but that clone 2 was also capable of expressing a second S6 kinase isoform of M_r 85,000. To test this possibility, anti-peptide antibody was generated against a unique sequence present in the 5′ coding region of clone 2. This antibody only immunoprecipitated the M_r 85,000 band from the translation products generated from clone 2, and this reaction is blocked by competing peptide (lanes 5 and 6). These results demonstrated that the M_r 85,000 band corresponded to the 525-amino acid polypeptide encoded by clone 2 and indicated the existence of an isoform of $p70^{s6k}$, referred to here as $p85^{s6k}$ to distinguish it from the p90 S6 kinase family (1, 2).

Activated $p85^{s6k}$ in Cycloheximide-Treated Rat Liver. From the results described above, the question of whether this M_r 85,000 isoform of S6 kinase was present *in vivo* and whether

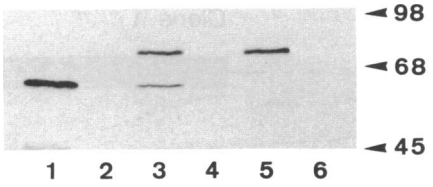

FIG. 4. Immunoprecipitation of *in vitro* translation products. Clone 1 translation products immunoprecipitated with anti-$p70^{s6k}$ antibody in the absence (lane 1) or presence (lane 2) of competing peptide. Clone 2 translation products immunoprecipitated with anti-$p70^{s6k}$ antibody or anti-$p85^{s6k}$ antibody in the absence (lanes 3 and 5, respectively) or presence (lanes 4 and 6, respectively) of competing peptide. Numbers on right are $M_r \times 10^{-3}$.

it was activated by mitogens arose. In preparations of purified $p70^{s6k}$ from cycloheximide-treated rat liver, a number of minor bands could be detected by silver staining (14, 15). To determine whether any of these bands was equivalent to $p85^{s6k}$, we examined a highly purified fraction of $p70^{s6k}$ by Western blot analysis with either the anti-$p70^{s6k}$ antibody or the specific anti-$p85^{s6k}$ antibody. The anti-$p70^{s6k}$ antibody recognized a major band at and a minor band at M_r 85,000 (Fig. 5*A*, lane 1), whereas the anti-$p85^{s6k}$ antibody only detected the M_r 85,000 band (lane 3). In both cases, detection of either band was blocked by the competing peptide (lanes 2 and 4), confirming that the M_r 85,000 band is equivalent to $p85^{s6k}$. Since the anti-$p85^{s6k}$ antibody exclusively recognizes this isoform of the S6 kinase on Western blots, it could specifically immunoprecipitate this form of the kinase. To test this possibility, the same highly purified fraction of the $p70^{s6k}$ was first immunoprecipitated with either the anti-$p70^{s6k}$ or the anti-$p85^{s6k}$ antibody and the immunoprecipitates were then incubated with [^{32}P]ATP (Fig. 5*B*, lanes 1 and 3). The results showed that the anti-$p70^{s6k}$ antibody immunoprecipitated both the major $p70^{s6k}$ as well as the $p85^{s6k}$ and that the anti-$p85^{s6k}$ antibody is specific for $p85^{s6k}$. In each case, competing peptides blocked immunoprecipitation of the corresponding phosphorylated kinase (lanes 2 and 4). Furthermore, the anti-$p85^{s6k}$ antibody immunoprecipitated S6 kinase activity, which was blocked by competing peptide (Fig. 5*C*). The results demonstrate that $p85^{s6k}$ is present in the livers of cycloheximide-treated rats, autophosphorylates, and has S6 kinase activity.

Activation of $p85^{s6k}$ in Swiss 3T3 Cells. Purification of the S6 kinase from Swiss 3T3 cells after mitogenic stimulation with either serum or epidermal growth factor has revealed only $p70^{s6k}$ (3, 5, 7, 15). However, a Northern blot of poly(A)$^+$ RNA from mouse cells exhibited a pattern very similar to that seen for rat liver (data not shown), raising the possibility that $p85^{s6k}$ could contribute to the overall S6 kinase activity in stimulated cell extracts. To test this possibility, cell extracts were prepared from either quiescent or 60-min serum-

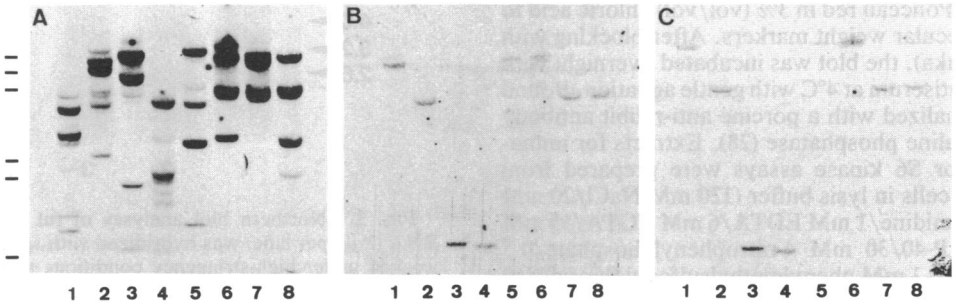

FIG. 3. Southern blot analyses of genomic rat liver DNA. Genomic DNA was digested in each lane as follows: 1, *Pst* I; 2, *Eco*RI; 3, *Hin*dIII; 4, *Eco*RI/*Hin*dIII; 5, *Nco* I; 6, *Bam*HI; 7, *Xba* I; 8, *Xba* I/*Bam*HI and hybridized with specific DNA probes. (*A*) Common coding region (nt 122–1706). (*B*) The 5′ end of clone 2 (nt 14–97). (*C*) Common coding region (nt 122–376) (18). Lines on the left correspond to DNA size markers (top to bottom) 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 kb.

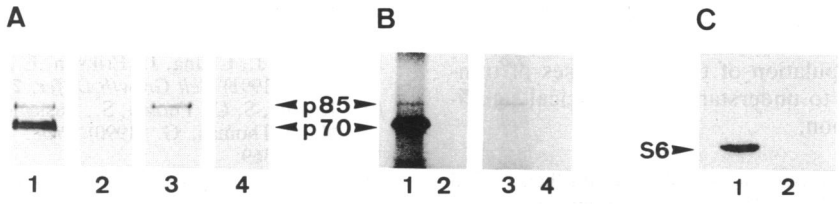

FIG. 5. Rat liver p85^{s6k}. (*A*) Western blot analysis of purified rat S6 kinase using the anti-p70^{s6k} antibody or the anti-p85^{s6k} antibody in the absence (lanes 1 and 3, respectively) or presence (lanes 2 and 4, respectively) of competing peptide. (*B*) Autoradiogram of autophosphorylated S6 kinase immunoprecipitated by anti-p70^{s6k} antibody or anti-p85^{s6k} antibody in the absence (lanes 1 and 3, respectively) or presence (lanes 2 and 4, respectively) of competing peptide. (*C*) Activity of rat S6 kinase immunoprecipitated by anti-p85^{s6k} antibody in the absence (lane 1) or presence (lane 2) of competing peptide.

stimulated Swiss 3T3 cells and assayed for S6 kinase activity directly or after immunoprecipitation with either anti-p70^{s6k} antibody or anti-p85^{s6k} antibody. The results showed that the S6 kinase activity in whole cell extracts was increased between 5- and 10-fold after serum stimulation (Fig. 6, lanes 1 and 2). A similar level of activation was obtained after immunoprecipitation with the anti-p70^{s6k} antibody and this activity was blocked by competing peptide (lanes 3–6). Use of the anti-p85^{s6k} antibody also revealed an increase in S6 kinase activity, which was specifically blocked by competing peptide (lanes 7–10). Thus, the p85^{s6k}, although present in apparently smaller amounts than p70^{s6k}, obviously contributed measurably to overall S6 kinase activity.

DISCUSSION

The results presented here demonstrated that two rat cDNA clones differing at their 5′ ends encoded two isoforms of p70^{s6k} that have S6 kinase activity. Furthermore, it was shown that these two clones were transcribed from a single gene and that the rat genome contains no full-length *s6k* pseudogenes. It should be noted, however, that both the isolation of clones 1 and 2 (18, 19) and the Northern and Southern blot analyses were carried out under high-stringency conditions; thus, the existence of a larger *s6k* gene family, although unlikely, cannot be excluded. Using Southern blot analysis, the *s6k* gene would appear to be quite large, ≈25 kb, which is consistent with a large (6.0 kb) transcript (Fig. 2). That the 5′ coding sequence of clone 2 appeared contiguous with the coding region common to both clones argues that the 5′ untranslated region of clone 1 is located further upstream in the *s6k* gene. Thus, clone 1, and most likely clone 2, would be generated by alternative splicing as well as the use of different polyadenylylation sites consistent with the Northern blot analysis depicted in Fig. 2. However, the possibility of alternative promotor usage for the production of the 2.5-kb transcript cannot be excluded. The cloning and sequencing of the 5′ end of the *s6k* gene should resolve this question.

In earlier studies from this laboratory describing the purification of the p70^{s6k} from either Swiss 3T3 cells or the livers of cycloheximide-treated rats, we never observed the p85^{s6k} by either silver staining or autophosphorylation (14, 15, 29). In contrast, Price *et al.* (13) have reported a protein of very similar size from the livers of cycloheximide-treated rats, which copurified with p70^{s6k} and was detectable by phosphorylation of purified fractions incubated with [^{32}P]ATP. This protein, however, was not labeled by azido-ATP, as was p70^{s6k}, nor did V8 peptide maps of the ^{32}P-labeled protein correspond to those from the p70^{s6k}, leading them to conclude that this material was a contaminant. Using specific antibodies that do not crossreact with p70^{s6k}, we clearly demonstrate the presence and activity of p85^{s6k} in the livers of cycloheximide-treated rats. By silver staining these fractions on SDS/polyacrylamide gel, the p85^{s6k} was barely detectable. However, the specific antibody to p85^{s6k} has allowed us to screen a number of earlier S6 kinase preparations. In all preparations tested p85^{s6k} was present, although at variable levels, arguing that p85^{s6k} was differentially removed during purification of p70^{s6k}.

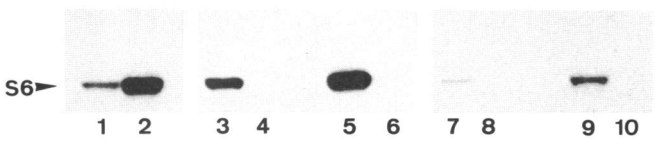

FIG. 6. S6 kinase activity in serum-stimulated Swiss 3T3 cells. Cell extracts from quiescent (lanes 1, 3, 4, 7, and 8) or serum-stimulated (lanes 2, 5, 6, 9, and 10) cells were assayed directly (lanes 1 and 2) or after immunoprecipitation with the anti-p70^{s6k} antibody (lanes 3 and 5) or the p85^{s6k} antibody alone (lanes 7 and 9) or in the presence of corresponding competing peptide (lanes 4, 6, 8, and 10). The amount of cell extract used in lanes 3–6 was 50 μg and the amount in lanes 7–10 was 200 μg.

The results shown in Fig. 6 also show that p85^{s6k} is activated by serum stimulation of quiescent Swiss 3T3 cells. The amount of total activity attributable to p85^{s6k} could only be roughly assessed, but it appeared to be low. If the S6 kinase is highly specific for 40S ribosomal protein S6 (17, 29), it raises the question of the purpose of this new isoform. One possibility would be that the N terminus of the enzyme targets the p85^{s6k} to a cell compartment different from that of p70^{s6k}. It has recently been demonstrated by Franco and Rosenfeld (30) that phosphorylated S6 exists in the nucleus, although it was not possible to determine whether it was phosphorylated in this compartment or transported there. The amino terminus of p85^{s6k} has two features that suggest it could be targeted to the nucleus (31). First, it contains a hexapeptide extending from amino acids 2 to 7, which are very similar to a sequence identified in the human immunodeficiency virus type 1 transactivator protein REV (amino acids 40–45) (31–33), which act as a nuclear/nucleolar localization signal. Second, it harbors a casein kinase II site at amino acid position 40 that is very similar to other proteins targeted to the nucleus (34). In the case of simian virus 40 large tumor antigen, it was shown that phosphorylation of this site controls the rate of nuclear import (34). Indeed, preliminary studies with affinity-purified antibodies indicate the p85^{s6k} is targeted to the nucleus (C.R., N. Lamb, and G.T., unpublished data).

During preparation of this manuscript, a report describing cloning and expression of the homologous human p70^{s6k} and p85^{s6k} appeared (35). The human p70^{s6k} shared 100% identity with the coding sequence of rat p70^{s6k} (18), whereas the human p85^{s6k} had two amino acid differences: an aspartic acid instead of histidine at position 16 and a proline instead of arginine at position 367 (19). These authors also reported the presence of a larger protein in purified preparations of p70^{s6k} from the livers of cycloheximide-treated rats by Western blot analysis (34). Whether this larger protein is equivalent to p85^{s6k} could not be ascertained, since the antibody used was not specific for p85^{s6k}. Perhaps still more intriguing

than the existence of $p85^{s6k}$ is the apparent evolutionary conservation of two different $p70^{s6k}$ transcripts. Isolation of the *s6k* gene and manipulation of the two classes of transcripts will be required to understand the biological significance of this phenomenon.

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