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Characterization of a rat liver factor that inhibits initiation of protein synthesis in rabbit reticulocyte lysates

(phosphorylation of Met-tRNA$_f$ binding factor/translational inhibitor)

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ABSTRACT **Protein synthesis in rabbit reticulocytes and their lysates is regulated by heme. In heme-deficient reticulocyte lysates, protein synthesis proceeds at the initial rate for several minutes and then declines abruptly. Inhibition of protein synthesis is due to the activation of a heme-regulated translational inhibitor (HRI) which blocks the initiation of protein synthesis. Addition of the isolated HRI to hemin-supplemented lysates causes inhibition of initiation similar to that observed in heme-deficiency. HRI has been shown to be a protein kinase that specifically phosphorylates the Met-tRNA$_f$ binding factor (eIF-2). We have isolated an inhibitor (LI) of protein chain initiation from rat liver which displays properties similar to those of HRI: (*i*) the chromatographic behavior of LI on DEAE-Sephadex, DEAE-cellulose, and phosphocellulose is similar to that of HRI; (*ii*) both LI and HRI inhibit protein chain initiation in rabbit reticulocyte lysates with the same kinetics of inhibition—i.e., an initial period of synthesis for several minutes at the control rate followed by an abrupt decline in the rate of initiation; (*iii*) both inhibitions are prevented or reversed by eIF-2; (*iv*) GTP (2 mM) prevents, and ATP (2 mM) potentiates, the inhibition of protein synthesis induced by either inhibitor; (*v*) LI is associated with a protein kinase that also phosphorylates the 38,000-dalton subunit of eIF-2. These findings indicate that a mechanism for the regulation of protein synthesis similar to that found in rabbit reticulocytes may be present in rat liver.**

Protein synthesis in rabbit reticulocytes and rabbit reticulocyte lysates is regulated by heme (1–3). In reticulocyte lysates with optimal concentrations of added hemin, protein synthesis is maintained at the initial rate for a prolonged period (2–6). In heme-deficient lysates, protein synthesis proceeds at an initial rate for several minutes and then declines abruptly, yielding characteristic biphasic kinetics. Inhibition of protein synthesis is due to the activation of a translational inhibitor (4–6) which acts at the level of protein chain initiation (2–10). The activation of this heme-regulated inhibitor (HRI) [also called heme-controlled repressor by Rabinovitz and his associates (11)] does not require protein synthesis (4–6).§ The characteristics of the inhibition induced by the addition of purified HRI to heme-supplemented lysates are similar to those induced by heme deficiency: (*i*) biphasic kinetics of inhibition (12, 13); (*ii*) disaggregation of polyribosomes with a concomitant increase in 80S ribosomes (12, 14, 15); (*iii*) reversal of inhibition by the Met-tRNA$_f$ binding factor (eIF-2) (12, 13, 15, 16); (*iv*) reversal of inhibition by GTP (17); and (*v*) potentiation of inhibition by ATP (17). Recent findings indicate that HRI is a protein kinase that specifically phosphorylates the 38,000-dalton subunit of eIF-2 (18–22).

Several reports have shown that hemin enhances or maintains protein synthesis in nonerythroid cells such as Krebs II ascites cells (23, 24), HeLa cells (25), and blood platelets (26). A macromolecular inhibitor with properties similar to those of HRI has been isolated from Ehrlich ascites tumor cells (27). These findings suggest that hemin may play a regulatory role in nonerythroid cells and raise the possibility that inhibitors similar to the reticulocyte inhibitor are present in nonerythroid tissues. In this report we describe an inhibitor of protein chain initiation isolated from rat liver. The rat liver inhibitor (LI) displays inhibitory and chromatographic properties similar to those described for HRI. More significantly, the LI is associated with a protein kinase that also phosphorylates the 38,000-dalton subunit of eIF-2. A preliminary account of these studies has been reported previously (28).

MATERIALS AND METHODS

The materials utilized in these studies were obtained from the following sources: cycloheximide and hemin chloride from Calbiochem; DEAE-Sephadex A50 from Pharmacia Fine Chemicals; DEAE-cellulose DE52 and phosphocellulose P11 from Whatman; and [γ-^{32}P]ATP and L-[^{14}C]leucine (327 mCi/mmol) from New England Nuclear.

Protein Synthesis in Rabbit Reticulocyte Lysates. Reticulocyte lysates were prepared from 2-kg male New Zealand White rabbits made anemic by the injection of acetylphenylhydrazine as described previously (6). The composition of incubation mixtures for measurement of protein synthesis has been described (6, 13). Protein synthesis was assayed by the incorporation of [^{14}C]leucine into proteins in reaction mixtures (25–30 μl) containing 0.2 μCi of [^{14}C]leucine and 10 μM hemin unless otherwise noted. HRI was prepared as described (20). The activity of LI was assayed at each stage of purification by its inhibitory effect in a standard reticulocyte lysate incubation mixture. In general, the extent of inhibition was measured by the amount of LI required to produce 50% inhibition in 30 min.

Preparation of the Rat Liver Translational Inhibitor. LI was isolated from rat liver postribosomal extracts based on the purification scheme previously developed for the purification of HRI (20). All steps were carried out at 4°. Livers (150 g total wet weight) from 53 Sprague-Dawley male rats, starved for 24 hr, were finely minced and then washed in buffer containing 50 mM Tris·HCl (pH 7.6), 75 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 250 mM sucrose. Extracts

Abbreviations: HRI, heme-regulated inhibitor; eIF-2, initiation factor which binds Met-tRNA$_f$; LI, inhibitor from rat liver; cAMP, adenosine 3′:5′-cyclic monophosphate.

§ Because this heme-regulated inhibition occurs at the level of translation and not of transcription, we believe that the term "inhibitor" is more appropriate than "repressor."

were prepared by gentle homogenization in several volumes of the same buffer. A ribosome-free supernate (S150) was obtained by centrifugation at 150,000 × *g* for 3 hr. Solid ammonium sulfate was added to the S150 supernate to bring it to 40% saturation, and the preparation was then left overnight in an ice bath. The precipitate was collected by centrifugation and dissolved in buffer A [5 mM *N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes), pH 7.2/200 mM KCl] and dialyzed against the same buffer.

The crude protein extract (1636 mg) was first separated by chromatography on a DEAE-Sephadex A50 column (2.6 × 100 cm, equilibrated in buffer A) with a stepwise KCl gradient (0.2, 0.25, and 0.4 M) in buffer A. Inhibitory activity was in the protein fraction that was eluted between 250 and 400 mM KCl (not shown). Fractions were concentrated and dialyzed against buffer B (3 mM Hepes, pH 7.2/70 mM KCl/0.05 mM EDTA/0.1 mM dithiothreitol). A 75-mg portion of the fraction was then subjected to chromatography on a DEAE-cellulose 52 column (0.9 × 30 cm) in buffer B with a linear KCl gradient (70–400 mM). Inhibitory activity was eluted as a major peak with the bulk protein between 150 and 175 mM KCl; the protein peak was divided into three fractions (D1, D2, D3) (Fig. 1*A*) which were concentrated separately; fraction D2 contained most of the inhibitory activity. Prior to phosphocellulose chromatography, fraction D2 was first dialyzed against buffer C (50 mM, Tris·HCl, pH 7.6/40 mM KCl) followed by a second extensive dialysis against buffer D (50 mM Tris·HCl, pH 6.7/40 mM KCl). A 23-mg sample was subjected to chromatography on a phosphocellulose column (0.9 cm × 22 cm) in buffer D [containing 10% (vol/vol) glycerol] with a stepwise KCl gradient (40, 100, 200, and 400 mM KCl). Inhibitory activity was eluted in two peaks at 100 mM KCl (PC2a) and 200 mM KCl (PC2) (Fig. 1*B*).

Preparation of the Translational Inhibitor from Perfused Rat Livers. Male Sprague-Dawley rats (135–145 g) were given 10 units of heparin by subcutaneous injection and were then decapitated. The abdomen was opened through a midline incision and the liver was perfused through the portal vein with ice cold Krebs–Ringer buffer at a rate of 30 ml/min for 20 min, followed by perfusion with 100 ml of ice-cold buffer containing 50 mM Tris·HCl (pH 7.6), 75 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 250 mM sucrose. Perfused livers from seven rats were obtained. The procedures for the preparation of ribosome-free supernate and LI were as described in the preceding section except that the chromatography on DEAE-cellulose was omitted. The LI prepared from extracts of perfused livers manifested the same chromatographic behavior on DEAE-Sephadex and phosphocellulose as that prepared from extracts of unperfused liver.

Determination of Concentration of Protein. Protein concentration was determined by the method of Lowry *et al.* (29), with bovine serum albumin as a standard.

Analysis of the Distribution of Polyribosomes. The polyribosome distribution in incubation mixtures (90 μl) was analyzed in sucrose density gradients (16–50%) in buffer E (20 mM Tris·HCl, pH 7.6/80 mM KCl/2 mM magnesium acetate). Aliquots (15 μl) of the incubation mixture were removed, diluted with 70 μl of cold buffer E, and layered on 5-ml gradients. The gradients were centrifuged at 4° in a SW 50.1 rotor at 38,000 rpm for 1 hr, and the optical density profile was monitored in an ISCO density gradient monitor at 254 nm. The fractions from the gradients were analyzed for the distribution of nascent polypeptide chains according to Darnbrough *et al.* (30).

Determination of RNase Activity. RNase activity in the LI preparations was assayed by the extent of polyribosome degradation in lysates incubated in the presence of 0.25 μM cycloheximide and in the absence of added amino acids, ATP, GTP, creatine phosphate, and creatine phosphokinase.

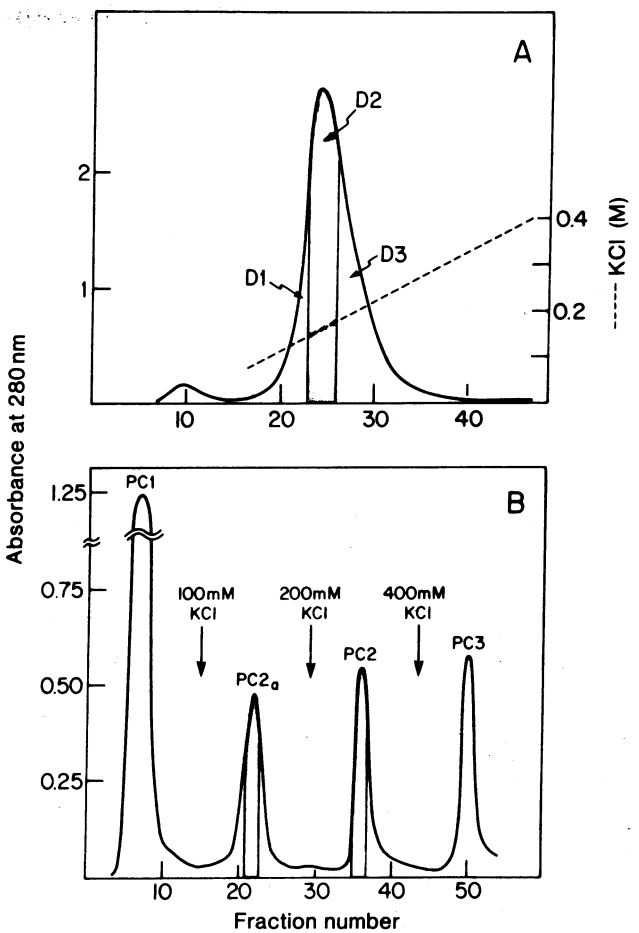

FIG. 1. Chromatography of LI on DEAE-cellulose (*A*) and phosphocellulose (*B*). The hatched areas represent inhibitor activity as determined in reticulocyte lysates. The details are described in *Materials and Methods.*

Preparation of eIF-2. The initiation factor eIF-2 which forms a ternary complex with Met-tRNA$_f$ and GTP (31–37) was isolated by chromatography of a 0.5 M KCl reticulocyte ribosomal salt wash on DEAE-cellulose and phosphocellulose (33). One microgram of the initiation factor preparation bound 0.44 pmol of Met-tRNA$_f$ in a ternary complex with GTP.

Assay for Protein Kinase Activity. Protein kinase activity was assayed by the covalent incorporation of the γ-phosphoryl group of [γ-^{32}P]ATP into calf thymus histone II, 40S ribosomal subunits, or eIF-2 as previously described (18).

RESULTS AND DISCUSSION

Chromatographic Properties of LI. Fractionation of rat liver postmicrosomal extracts (S150) yielded a partially purified preparation of LI that blocked protein synthesis initiation in hemin-supplemented reticulocyte lysates. LI displayed essentially the same chromatographic behavior as did HRI (20). LI was precipitated from a rat liver S150 extract with ammonium sulfate at 0–40% saturation. The inhibitor was eluted between 250 and 400 mM KCl from DEAE-Sephadex A50 (results not shown) and between 150 and 175 mM KCl from DEAE-cellulose (fraction D2, Fig. 1*A*). Chromatography on phosphocellulose yielded two activity peaks, one at 100 mM KCl (PC2a) and the other at 200 mM KCl (PC2) (Fig. 1*B*).

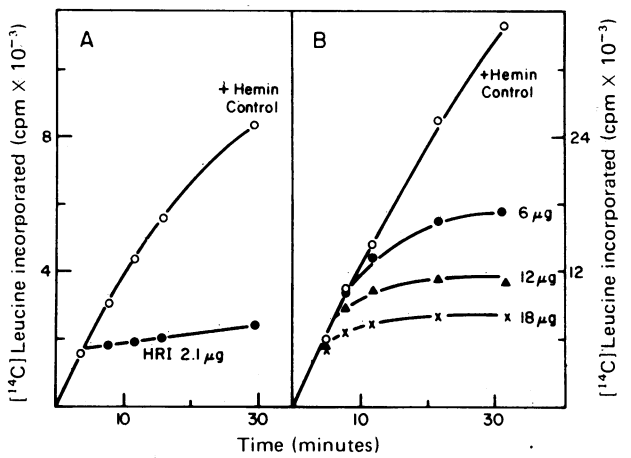

FIG. 2. Kinetics of inhibition of protein synthesis. Protein synthesis reaction mixtures (30 μl) containing 10 μM hemin were incubated at 30° with the indicated amounts of HRI (*A*) or LI (*B*) from perfused livers. At intervals, 5-μl aliquots were removed and assayed for [^{14}C]leucine incorporation as described in *Materials and Methods*.

Characteristics of the Inhibition Induced by LI. LI inhibited protein synthesis in reticulocyte lysates with kinetics similar to those induced by HRI. Protein synthesis proceeded at the initial control rates for several minutes followed by an abrupt decline in the rate (Fig. 2). As with HRI (12), the degree of inhibition increased with increasing concentrations of LI, and an initial period of synthesis, which comprised several reinitiation cycles prior to the onset of inhibition, was maintained even at relatively high concentrations of added LI.

The inhibition of protein synthesis in heme-supplemented lysates by LI was accompanied by a disaggregation of polyribosomes into 80S ribosomes, as in heme deficiency (Fig. 3). In order to eliminate the possibility that polyribosome disaggregation was due to RNase activity in the LI preparation, hemin-supplemented lysates were incubated in the presence of cycloheximide and LI; no polyribosome degradation was observed during the incubation (results not shown). LI did not influence the rate or extent of polyuridylic acid-directed polyphenylalanine synthesis (results not shown). Therefore, the inhibition of protein synthesis by LI is not due to the hydrolysis of mRNA by nuclease activity or to the inhibition of elongation. These results are consistent with an inhibitory effect of LI on polypeptide chain initiation.

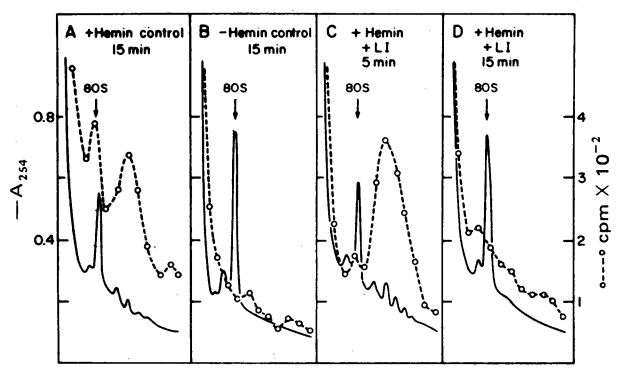

FIG. 3. Effect of LI on polyribosomes. Protein synthesis reaction mixtures (90 μl) containing 20 μM hemin were incubated at 30° with (95 μg) or without LI as described in *Materials and Methods*. At intervals, 15-μl aliquots were analyzed by sucrose density gradient centrifugation.

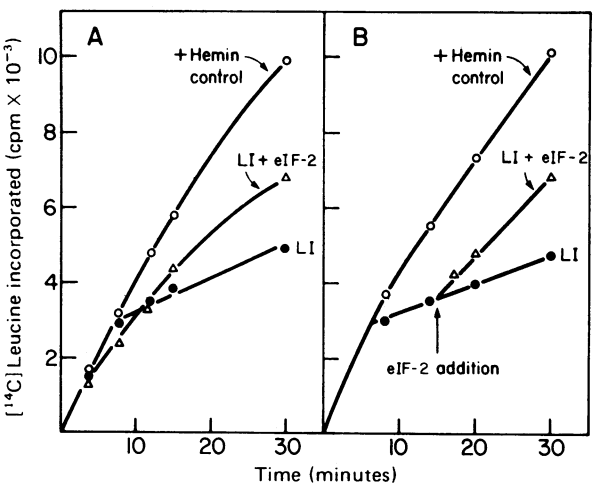

FIG. 4. Effect of eIF-2 on the inhibition of protein synthesis by LI. Protein synthesis reaction mixtures (30 μl) containing 20 μM hemin and 17 μg of LI (where indicated) were incubated at 30°. Where indicated, 6 μg of eIF-2 was added at zero time (*A*) or after 15 min of incubation (*B*). At intervals, 5-μl aliquots were removed and assayed for protein synthesis.

Reversal and Prevention of Inhibition by eIF-2. eIF-2 prevented inhibition of protein synthesis by LI in reticulocyte lysates when added at the start of incubation (Fig. 4*A*) and reversed inhibition when added after the onset of inhibition (Fig. 4*B*). The preventive or restorative effect of eIF-2 on the inhibition caused by LI was similar to that observed with HRI (12, 13, 15–17).

Effects of GTP and ATP on Inhibition Caused by LI. GTP (2 mM) partially blocked inhibition by LI, and ATP (2 mM) potentiated inhibition of protein synthesis by LI in reticulocyte lysates (Table 1). Similar effects of these nucleotides have been described in heme-deficient lysates and in hemin-supplemented lysates inhibited by HRI (17, 38).

Association of a Protein Kinase with LI. Protein kinase activities were present in all fractions during purification of LI. Through the DEAE-cellulose stage of purification, LI fractions contained an active adenosine 3′:5′-cyclic monophosphate (cAMP)-dependent protein kinase activity that extensively phosphorylated calf thymus histone II (Table 2). However, chromatography of this fraction on phosphocellulose yielded two protein kinase activities. The cAMP-dependent protein kinase activity appeared in the PC1 fraction which had no inhibitory activity, and a cAMP-independent protein kinase ac-

Table 1. Effects of ATP and GTP on the inhibition of protein synthesis induced by LI in reticulocyte lysates

LI	Addition	cpm	Inhibition, %
−	None	23,375	—
+	None	10,402	45
+	2 mM ATP	7,161	69
+	2.2 mM GTP	17,126	27

Reaction mixtures (28 μl) were prepared as described in *Materials and Methods* except that ATP was omitted and Mg^{2+} concentration was 1 mM; when ATP or GTP was added, equimolar concentrations of Mg^{2+} were also added. Where indicated, LI (22.5 μg of PC2a) and nucleotides were added at the start of incubation. The values in the table represent hot trichloroacetic acid-insoluble radioactivity in 5-μl aliquots after 60 min of incubation at 30°.

Table 2. Distribution of protein kinase activity during purification of LI

Fraction	Amount, μg	Inhibitory activity	γ-^{32}P incorporated into histone, pmol −cAMP	γ-^{32}P incorporated into histone, pmol +cAMP
D1	12	−	1	10
D2	12	+	7	>50
D3	14	−	4	>15
PC1	3	−	8	>90
PC2a	6	+	5	7
PC2	6	+	4	10
PC3	9	−	4	14

Reaction mixtures (25 μl) contained 25 mM Hepes (pH 7.15), 4 mM magnesium acetate, 25 μg of calf thymus histone II, 20 μg of bovine serum albumin, and 188 μM [γ-^{32}P]ATP adjusted to a final specific activity of 2220 cpm/pmol. The protein fractions represent the DEAE-cellulose (D) and phosphocellulose (PC) fractions indicated in Fig. 1. Incubation was at 30° for 15 min; hot trichloroacetic acid-insoluble radioactivity was determined as previously described (18).

tivity eluted with LI in the PC2a and PC2 fractions. A similar distribution of protein kinase activities on phosphocellulose has been observed in the course of purification of HRI (18, 20).

The noninhibitory fraction (PC1), containing a cAMP-dependent protein kinase, phosphorylated a polypeptide of the 40S ribosomal subunit that migrated as a 31,000-dalton component in sodium dodecyl sulfate/acrylamide gel electrophoresis (Fig. 5). The inhibitory LI fraction (PC2), which contained a cAMP-independent protein kinase, phosphorylated the small 38,000-dalton subunit of eIF-2. The same substrate specificities have been found for the two protein kinase activities of the corresponding phosphocellulose fractions in the purification of HRI (18). The finding that the protein kinase activity of LI phosphorylates the small polypeptide of eIF-2 provides additional evidence for the similarity of LI to HRI. As we have noted earlier (18), however, the relationship, whether direct or indirect, of the phosphorylation of eIF-2 to the mechanism of inhibition induced by heme-deficiency, HRI, or LI remains to be determined.

The amounts of LI isolated from rat liver are relatively low compared to the amounts of HRI obtained from reticulocytes. For the most purified LI preparation (obtained from perfused livers), 6 μg of protein was required to inhibit protein synthesis by 50% as compared to 0.1 μg of HRI at the same stage of purification. This difference may simply reflect a greater amount of inhibitor per unit weight in the reticulocyte and might raise the possibility that LI activity obtained from unperfused livers may be derived from contaminating erythroid cells. Our results with the isolation of LI from perfused livers, from which all blood had been removed, make this possibility unlikely.

The effect of heme in promoting protein synthesis has been demonstrated in Krebs ascites tumor cells (23, 24), in blood platelets (26), and in HeLa cell extracts (25). In addition, an inhibitor that resembles HRI has been found in Ehrlich ascites tumor cells (27). These various findings in erythroid and nonerythroid cells prompt the suggestion that eukaryotic protein synthesis is regulated by inhibitors with protein kinase activity. In reticulocytes this mechanism is regulated by heme, and it is possible that heme has a similar physiologic role in eukaryotic cells generally. We recognize, however, that it is quite possible that agents other than heme may also regulate this inhibitory mechanism, especially in nonerythroid cells.

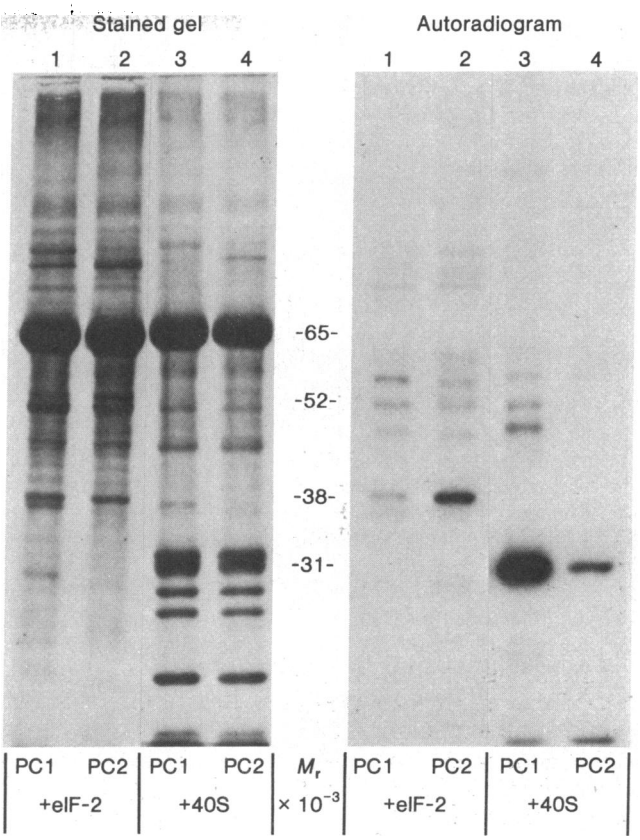

FIG. 5. Substrate specificity of the protein kinase activity in the LI preparation. The protein kinase activities of fractions PC1 and PC2 (Fig. 1*B*) were determined with eIF-2 and 40S subunits as substrates. Protein kinase mixtures (20 μl) were prepared as previously described (18). Where indicated, 4 μg of PC1 or 12 μg of PC2 was added. Exps. 1 and 2 contained 6 μg of eIF-2; Exps. 3 and 4 contained 0.27 A_{260} unit of 40S subunit plus 5 μM cAMP. After 10 min at 30°, samples were concentrated by precipitation in 8% trichloroacetic acid and electrophoresed in a sodium dodecyl sulfate/acrylamide slab gel (10% acrylamide/0.25% bisacrylamide with 4% spacer gel) as described (18). Autoradiography was carried out with RP/R54 Kodak x-ray film as previously described (18). (*Left*) Stained gel containing carrier bovine albumin in the heavy band at M_r = 65,000. (*Right*) Autoradiogram. M_r, molecular weight.

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