# Control of Eukaryotic Protein Synthesis by Phosphorylation

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Phosphorylated proteins as physiological effectors may have a much broader role than previously assumed. Many specific regulatory signals may activate one or more protein kinase that phosphorylate and thereby modulate the activity of key enzymes in various pathways. The sarcoma gene of avian sarcoma virus appears to code for a protein kinase that may disrupt normal differentiation to cause transformation in vitro and sarcoma induction in vivo [1]. Examples of other kinase systems are considered in a recent review article [2]. Some hormones and neurotransmitter substances are known to act through cAMP-dependent protein kinases to regulate carbohydrate and lipid metabolism as well as the characteristics of the cell's surface. However, recent work has focused on cAMP-independent protein kinases. Regulation of virus assembly provides a clear example of a cAMP-independent kinase system. Phosphorylation of viral core protein, p12, changes its ability to bind to viral RNA in either Rauscher leukemia virus [3] or simian sarcoma-associated virus [4]. Translational regulation of protein synthesis provides another example of a pathway that is regulated by cAMP-independent protein kinases. Here we will consider regulation of protein synthesis by cAMP-independent protein kinases with emphasis on examples in which a correlation between phosphorylation and inhibition of peptide initiation can be demonstrated.

## Eukaryotic Peptide Initiation

Regulation of eukaryotic protein synthesis is known to occur at steps of peptide initiation. This multi-step process is schematically outlined in Fig. 1. Though the general scheme is widely accepted, the actual function of most of the initiation factors involved is not yet understood nor is the exact point of GTP hydrolysis firmly established [cf. 6]. Most of the initiation factors consist of one peptide chain ranging in molecular weight from 15000 to 150000 [7]. Two initiation factors have a more complex structure; eIF-3 appears to be a high molecular weight complex consisting of 9–11 proteins [7,8] and eIF-2 contains 3 subunits with molecular weights of 34000, 48000 and 52000 daltons [7,9].

Phosphorylation has been reported for several eukaryotic initiation factors [10,11] and for proteins of the small as well as the large ribosomal subunit [12,13], but a correlation to or apparent change in function or activity has to

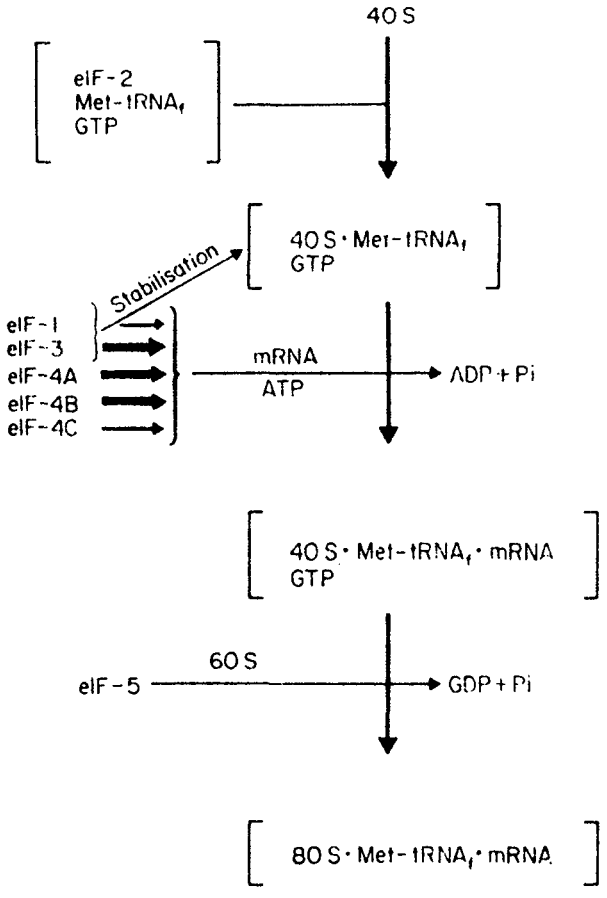


**Fig. 1.** Assembly scheme of the initiation complex. The relative importance of factors for mRNA binding is indicated by the thickness of the arrows. This figure is taken from ref. [5]

be demonstrated yet in these cases. However, phosphorylation of the smallest subunit of the initiation factor eIF-2 has been shown to inhibit protein synthesis as will be described in detail in the next paragraphs.

## Control of Protein Synthesis in Reticulocytes by Phosphorylation

As described in a previous contribution to this series [14], protein synthesis in reticulocytes is regulated by the availability of hemin. Incorporation of $^{14}C$ leucine into protein in a cell-free reticulocyte lysate system will stop after a few minutes of incubation unless hemin is added to the reaction mixture. Inhibition of protein synthesis in the absence of hemin appears to be achieved by preventing the formation of a stable 40S ribosomal subunit · Met-tRNA$_f$ initiation complex [15, 16]. An inhibitory protein(s) may be isolated from the reticulocyte postribosomal supernatant incubated for several hours in the absence of hemin [17] or briefly in the presence of N-ethylmaleimide [18, 19]. This protein (complex) has been called the hemin-controlled repressor (HCR; ref. [15, 17]).

Preparations of partially to highly purified HCR have been shown to contain protein kinase activities for the smallest subunit of the initiation factor eIF-2 [20–22] and for proteins of 40S ribosomal subunits [21]. The actual mechanism by which phosphorylation and inhibition is interlocked is an area of intensive studies at present. Also, the mechanism by which the protein kinase(s) are activated in the absence of hemin is not understood yet. Papers

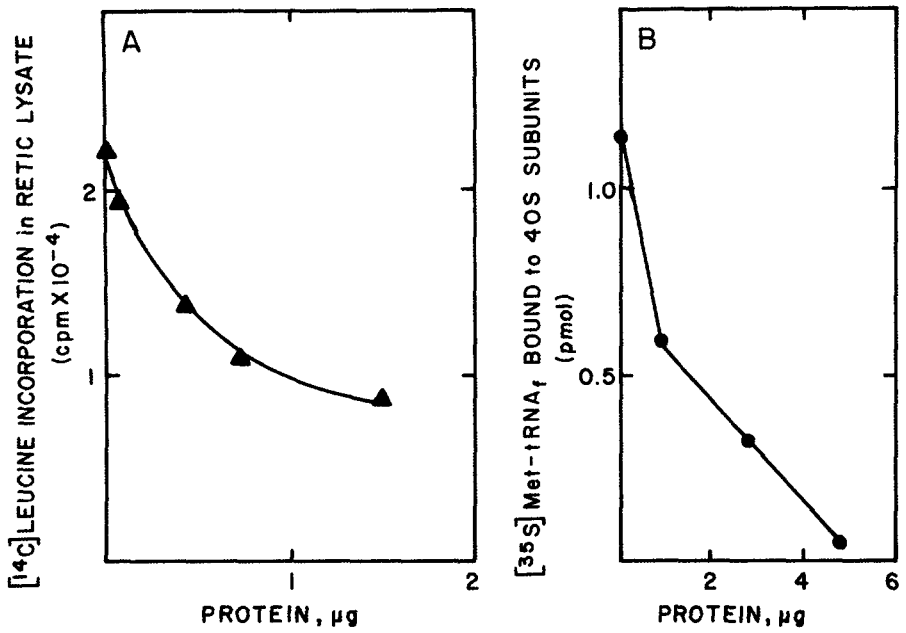


**Fig. 2.** Inhibition of protein synthesis in reticulocyte lysate and inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits by an HCR preparation.
*A* Protein Synthesis in rabbit reticulocyte lysates was carried out as described [25]. A 100-µl reaction mixture contained 20 µl lysate; $^{14}$C leucine was used at a specific radioactivity of 40 Ci/mole. HCR was added in the amounts shown.
*B* Binding of $^{35}$S Met-tRNA$_f$ (2 Ci/mmol) to reticulocyte 40S ribosomal subunits was performed as described previously [26]. The initiation factor eIF-2 (2.1 µg of protein) was preincubated with an HCR preparation in the amounts indicated and 0,5 mM ATP, then hexokinase and glucose was added for a second incubation. Finally, Met-tRNA$_f$ binding to 40S subunits was determined as detailed [26]

from S. Ochoa's laboratory claim the involvement of a cAMP-dependent protein kinase in the activation process (ref. [23] and earlier papers cited therein); Evidence is accumulating that cAMP is not involved in the activation of HCR [19, 19a].

Binding of Met-tRNA$_f$ to 40S ribosomal subunits that is dependent on eIF-2 and GTP can be measured directly in a partial reaction of peptide initiation [24]. We demonstrated that an HCR preparation will inhibit this reaction using reticulocyte 40S ribosomal subunits [25]. Furthermore, preincubation of either eIF-2 or reticulocyte 40S ribosomal subunits with an HCR preparation and ATP, followed by degradation of residual ATP, will inactivate these components for the subsequent Met-tRNA$_f$ binding reaction [26]. This reaction has to be carried out with a GTP-analog, GMP-P ($CH_2$)P, to prevent further phosphorylation. An experiment of this type is shown in Fig. 2B. HCR (protein kinase)-dependent phosphorylation of eIF-2 resulting in inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits (Fig. 2B) is compared with inhibition of $^{14}$C leucine incorporation in a cell-free reticulocyte lysate system by the same preparation of HCR (Fig. 2A). Whether phosphorylated eIF-2 is impaired for direct interaction with 40S subunits or for interaction with another (initiation) factor [27] has yet to be demonstrated.

## Translational Inhibitors from Uninduced and DMSO-Stimulated Friend Leukemia Cells: Comparison to the Reticulocyte HCR

Friend leukemia cells (FLC) are capable of undergoing partial erythroid differentiation by the addition of various organic compounds such as dimethylsulfoxide [28], butyric acid [29] or hexamethylene bisacetamide [30] to the growth medium.

Protein synthesis in uninduced FLC appears not to be regulated by hemin. The data in Fig. 3 indicate that hemin has no effect on the rate of protein synthesis in FLC that do not synthesize hemoglobin. Incorporation of $^3$H leucine into peptide chains was compared in the absence and presence of hemin. Desferal [31] was used in the control condition to remove trace amounts of free iron ions which may be present in the incubation medium. This compound itself showed no effect on protein synthesis (data not shown). FLC that had been treated with dimethylsulfoxide (DMSO) for 5 days synthesized hemoglobin as judged by their red color and benzidine staining (about 41% positive; viability over 85%). Fig. 3 indicates that protein synthesis in these hemoglobin producing cells is dependent on hemin. In its absence, $^3$H leucine incorporation over a period of 2 hr is reduced by about 40%.

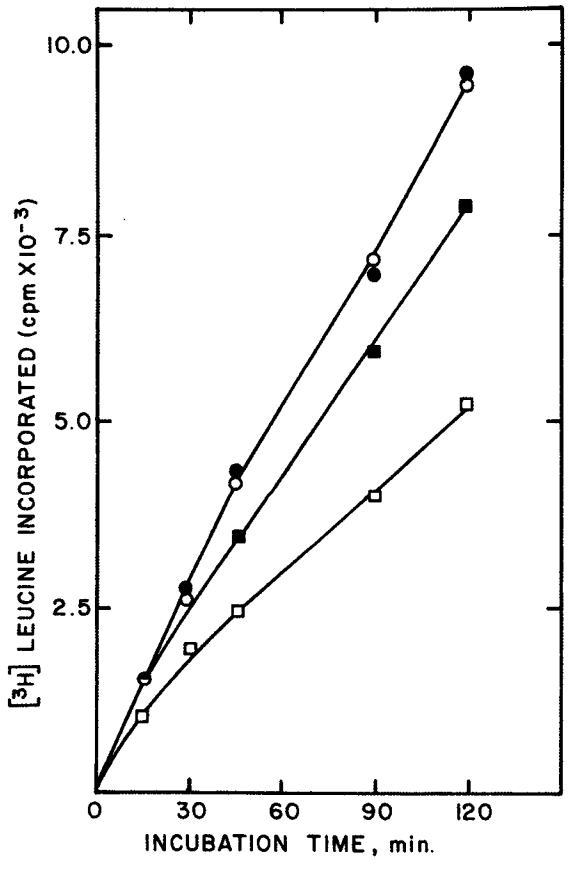


**Fig. 3.** Effect of hemin on $^3$H leucine incorporation in vivo in unstimulated or DMSO-stimulated FLC. Protein synthesis was determined by $^3$H leucine incorporation (30 μM, 8000 Ci/mol) in uninduced FLC or FLC that had been treated for 5 days with 1,5% DMSO (cf. [33]). About $3 \times 10^7$ cells in a final volume of 0,55 ml were incubated either with 10 μg hemin or 10 μg desferal (deferoxamine mesylate, Ciba Pharmaceutical Co., Summit, NJ). Aliquots of 20 μl were withdrawn at the indicated times, placed into 2.5 ml ice-cold NKM-solution and filtered through Millipore filters. These were washed with NKM, then with 5% trichloroacetic acid, and its radioactivity determined by liquid scintillation counting.

○——○ = uninduced FLC + desferal
●——● = uninduced FLC + hemin
□——□ = DMSO-stimulated FLC + desferal
■——■ = DMSO-stimulated FLC + hemin

A translational inhibitor has been isolated from uninduced FLC [32]. This inhibitor has functional similarities to HCR but differs in physical properties. Pretreatment of the postribosomal supernatant fraction from FLC was not necessary to detect inhibitory activity. In search for HCR-like inhibitors that

may be present in the inactivate form in the postribosomal supernatant of either uninduced or DMSO-treated FLC, these fractions were incubated with N-ethylmaleimide (NEM), a compound known to activate HCR [18,19]. Untreated or NEM-treated postribosomal fractions from uninduced or DMSO-stimulated FLC were processed in parallel and chromatographed on DEAE cellulose and phosphocellulose to partially purify inhibitory activity. Inhibitory activity was assayed in the reticulocyte cell-free lysate system. One unit of inhibitory activity is defined as the amount of protein that reduces $^{14}C$ leucine incorporation by 50% in the reticulocyte lysate system used. The results presented in Table 1 demonstrate that the inhibitor fraction from DMSO-induced FLC exhibits a higher specific activity after the postribosomal supernatant of these cells was incubated with NEM suggesting activation of an HCR-like translational inhibitor. An authenic HCR fraction from reticulocytes that was carried through the same chromatography steps showed 7–8 fold higher inhibitory activity when assayed under the same conditions (Table 1). The four different inhibitor fractions isolated from FLC as listed in Table 1 possess strong protein kinase activity for the smallest subunit of eIF-2 as shown in Fig. 4. Consequently, they inhibit the binding of Met-$tRNA_f$ to reticulocyte 40S ribosomal subunits as does HCR (cf. Fig. 2B). In Fig. 5 this inhibition of the Met-$tRNA_f$ binding reaction is compared with the inhibitor fractions isolated from uninduced and DMSO-stimulated FLC. In both cases the postribosomal supernatant was treated with NEM. The experiment was done in the same way as described in Fig. 2B: eIF-2 was incubated under protein kinase conditions with the inhibitor fraction and ATP, residual ATP was destroyed by the use of glucose and hexokinase. Then the activity of eIF-2 was measured in the Met-$tRNA_f$ binding reaction. The results (Fig. 5) show that eIF-2 is inactivated depending on the concentration of the inhibitor used. Furthermore, the inhibitor fraction derived from DMSO-stimulated FLC exhibit stronger inhibitory activity than the one from uninduced FLC. These findings are in agreement with the results shown above in Table 1.

**Table 1.** Inhibition of protein synthesis by different inhibitor preparations

| Source of Inhibitor Fraction | NEM Treatment | Specific Activity (units/mg) |
|---|---|---|
| Uninduced FLC | – | 88 |
| Uninduced FLC | + | 88 |
| DMSO-induced FLC | – | 90 |
| DMSO-induced FLC | + | 222 |
| Reticulocytes | – | 0 |
| Reticulocytes | + | 1904 |

Inhibitor fractions were prepared from the postribosomal supernatant by chromatography on DEAE cellulose and phosphocellulose as described [31]. Activity was assayed in the reticulocyte lysate system as outlined in the legend to Fig. 2

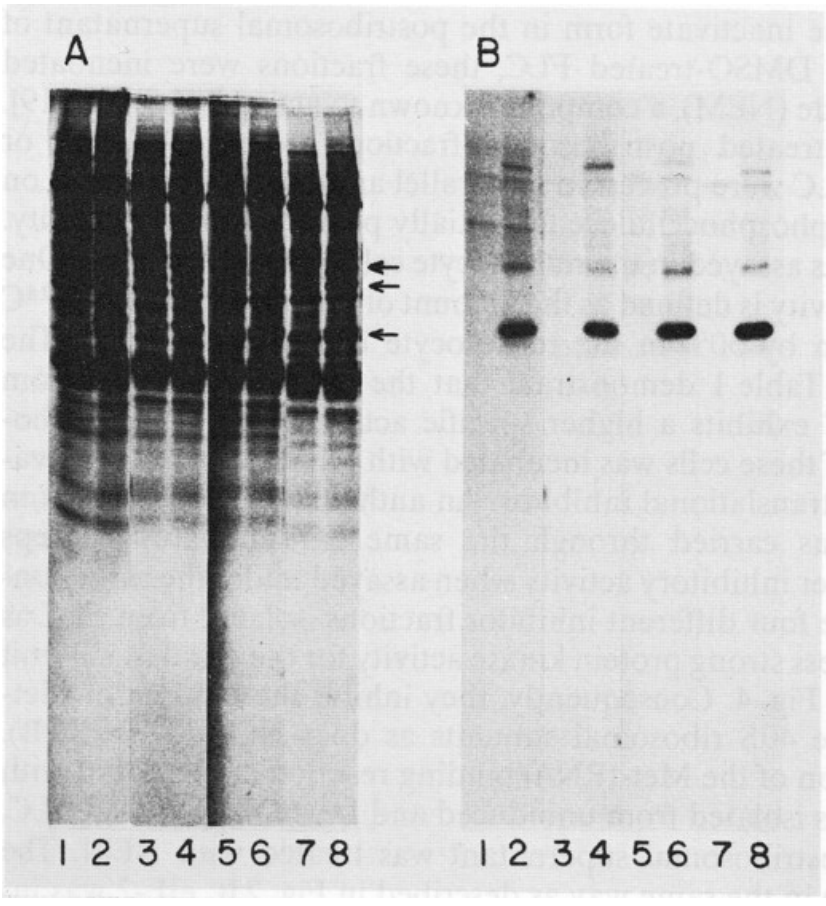


**Fig. 4.** Protein kinase activity of the FLC inhibitor fractions. Samples containing the different inhibitor fractions described in the text (2,5 units each) were incubated without or with 2.5 μg eIF-2 or 0.2 mM $^{32}$P ATP (650 Ci/mol) and analyzed by SDS-polyacrylamide gel electrophoresis [26].

*A* Shows the gel stained with Coomassie brilliant blue. The arrows indicate the positions of the 3 subunits of eIF-2.

*B* Shows the autoradiogram. The following samples were loaded on slots 1–8: 1 = 31 μg of untreated, uninduced FLC inhibitor; 2 = as 1, plus eIF-2; 3 = 31 μg of NEM-treated, uninduced FLC inhibitor; 4 = as 3, plus eIF-2; 5 = 28 μg of untreated, DMSO-induced FLC-inhibitor; 6 = as 5, plus eIF-2; 7 = 11 μg of NEM-treated, DMSO-induced FLC-inhibitor; 8 = as 7, plus eIF-2

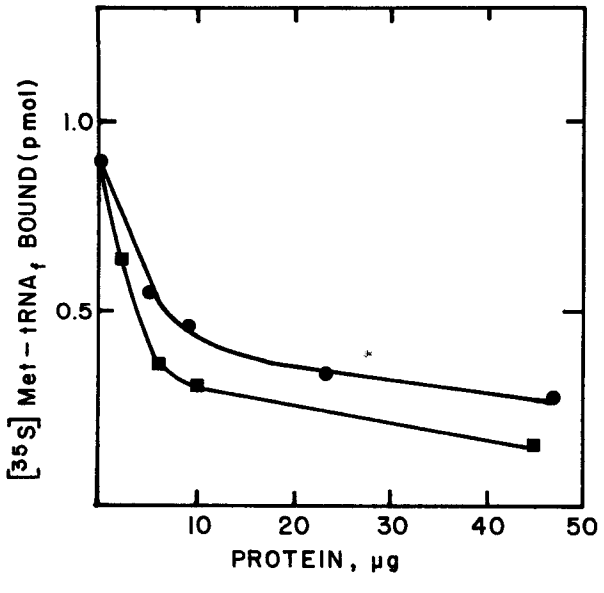


**Fig. 5.** Inhibition of Met-tRNA$_f$ binding to 40S ribosomal subunits by FLC inhibitors. The experiment was performed as described in the legend to Fig. 2B except that FLC inhibitors in the amount indicated substituted for HCR.

●——● = NEM-treated, uninduced FLC-inhibitor;

■——■ = NEM-treated, DMSO-induced FLC-inhibitor

## Discussion

Inhibition of peptide initiation is known to provide translational regulation in reticulocytes. This inhibition occurs at the step in which Met-tRNA$_f$ is bound to 40S ribosomal subunits and is mediated by a protein kinase(s) that phosphorylate the smallest subunit of the initiation factor eIF-2 and proteins of the 40S ribosomal subunit. Regulation of protein synthesis may be unique in reticulocytes (and other hemoglobin-synthesizing cells) in that the level of hemin controls the activity of these protein kinases.

Regulation of protein synthesis by phosphorylation (and counteracting phosphatases that have yet to be described) may be a more general principle. Inhibitory protein kinases that phosphorylate the smallest subunit of eIF-2 have been partially purified from FLC as detailed above and have been described to occur in rat liver [34] or Ehrlich ascites cells [35]. Also, a protein kinase that phosphorylates the same subunit of eIF-2 appears to be involved in the inhibition of protein synthesis observed after addition of double-stranded RNA to reticulocyte lysates [20] or to interferon-treated cell extracts [36–38].

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