Review Letter

CONTROL OF THE INITIATION OF PROTEIN SYNTHESIS IN MAMMALIAN CELLS

Sara A. AUSTIN and Michael J. CLEMENS

Department of Biochemistry, St George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, England

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1. Introduction

This review describes the current state of knowledge of the mechanisms by which the initiation of protein synthesis is controlled in mammalian cells. We have chosen to consider only translational control mechanisms, which limit the rates at which mRNAs are translated by the protein synthetic machinery, rather than transcriptional control mechanisms, by which the number and types of mRNAs available for translation are regulated, as the latter are beyond the scope of a short review.

The most extensively studied mammalian system and the one from which most knowledge of translational control mechanisms has been obtained is the reticulocyte and its corresponding cell-free system, the reticulocyte lysate. The first part of this review deals with the way in which the availability of haem regulates the rate of protein synthesis in the reticulocyte lysate. Other factors which can control protein synthesis in this system and the similarities between control mechanisms which operate under these circumstances and those which operate in haem deficiency are also discussed.

The second half of the review is concerned with the mechanisms which control rates of protein synthesis in mammalian cell types other than the reticulocyte, where cellular functions are less specialised and where the presence of a nucleus implies that translational control mechanisms might have a different role in the control of protein synthesis than in the non-nucleated reticulocyte. The similarities and differences between these types of systems are considered.

2. Initiation of protein synthesis in the reticulocyte lysate

Protein synthesis in the reticulocyte lysate is closely regulated by the supply of haem, as it is in whole reticulocytes [1,2]. In the presence of haem crude reticulocyte lysates synthesize proteins at almost the same rates as the whole cells, but in the absence of haem protein synthesis is rapidly shut off after a lag period, the length of which depends on the temperature of incubation [3,4]. The shut off in protein synthesis is associated with polysome breakdown and a reduction in the level of [40 S · Met-tRNA$_f^{Met}$] initiation complexes indicating a decline in the rate of initiation [5]. It is now well established that the effects of haem deficiency in the lysate are due to the formation of an inhibitor, known as the haem controlled repressor (HCR) or haem-regulated inhibitor (HRI) [5–7]. In the absence of haem this inhibitor is formed by conversion of an inactive proinhibitor to an active form [8]. This reaction is initially reversible by addition of haem but prolonged absence of the latter leads to a transition of the HCR to an irreversible form [9,10]. It is with this form of HCR that most work has been done.

The most purified preparations of HCR indicate 80 000–90 000 mol. wt on SDS–polyacrylamide gels [11]. Undoubtedly the most interesting property of this inhibitor is that is has protein kinase activity specifically directed towards the smallest subunit of eukaryotic initiation factor 2 (eIF-2) [11,12]. Factor eIF-2 binds the initiator Met-tRNA$_f$ in a ternary complex with GTP and is involved in [40 S · Met-tRNA$_f^{Met}$] initiation complex formation [13,14]. It now seems

certain that the phosphorylation of eIF-2 by HCR is associated with the decline in initiation observed in the absence of haem. The effects of HCR can be overcome by addition of exogenous eIF-2 to the lysate [15,16], and there is a good correlation between the effects of molecules which inhibit eIF-2 phosphorylation and their ability to stimulate protein synthesis in the haem-deficient lysate [11,17,18]. Furthermore, an antiserum to HCR which eliminates the effects of the latter on protein synthesis also stops the phosphorylation of eIF-2 [19]. However a major problem still exists in this otherwise consistent model since, contrary to expectations, purified eIF-2 is active in highly fractionated cell-free systems whether it is phosphorylated or not [11,20,21]. It is likely that, in such systems, eIF-2 does not recycle and only acts stoichiometrically rather than catalytically [20,22]. Thus it was suggested that phosphorylation of eIF-2 by HCR might allow one round of initiation per molecule of eIF-2 present, but would still block the ability of this factor to recycle [22]. However, it has recently been reported that both phosphorylated and unphosphorylated eIF-2 can initiate between three and five rounds of protein synthesis per molecule when added to a lysate [23]. In addition the same laboratory has isolated another factor from reticulocytes which reverses the effect of HCR on protein synthesis in lysates. This factor, called anti-inhibitor, is distinct from eIF-2 and has no effect on phosphorylation of eIF-2 by HCR [23]. A similar factor has been characterised in [24,25]. In the light of this evidence it has been suggested that inactivation of eIF-2 in haem-deficient lysates is caused by the reversible binding of that factor to a component, as yet unidentified, which is present in crude lysates, but which is lost on purification (both HCR and the anti-inhibitor being inactive with purified eIF-2). It is possible that phosphorylation of eIF-2 may be a requirement for this binding to occur and the role of the anti-inhibitor could then be to prevent such a reaction by itself binding to eIF-2 [23].

Several groups have reported the existence of factors which stimulate the activity of eIF-2 in fractionated cell-free systems and which enhance or restore the sensitivity of initiation complex formation to inhibition by HCR. The nature of these factors is summarized in table 1. Ochoa's group have purified a stimulatory activity from both *Artemia* (brine shrimp) and rabbit reticulocytes, called eIF-2 stimulatory protein (ESP). This enhances [eIF-2 · GTP · Met-tRNA$_f$] and 40 S

Table 1
Protein factors associated with eIF-2 which modulate its activity in vitro

	Source	Activity	Mol. wt	Ref.
ESP	Artemia and rabbit reticulocytes	Stimulates ternary complex formation and 40 S initiation complex formation. Effect is abolished by HCR	200 000	[26,27]
SF	Rabbit reticulocytes	Stabilizes ternary complexes. Required for HCR catalysed inhibition of ternary complex formation	250 000–300 000	[28]
eIF-2 stimulator (GDPase)	Rat liver	Stimulates ternary complex and 40 S initiation complex formation. Has GDPase activity	Above 200 000	[29]
CO-eIF-2A (CO-EIF-1)	Rabbit reticulocytes	Stimulates ternary complex formation. Binds to eIF-2. Increases rate of Met-tRNA$_f^{Met}$ binding to 40 S subunits	25 000 and 200 000 forms	[30,31]
CO-eIF-2B (TDF)	Rabbit reticulocytes	Dissociates ternary complexes. Stimulates AUG-dependent binding of Met-tRNA$_f^{Met}$ to 40 S subunits	450 000	[32]
CO-eIF-2C (eIF-2B)	Rabbit reticulocytes	Relieves Mg^{2+} inhibition of ternary complex formation. Does not relieve GDP inhibition of ternary complex formation	–	[35]
SRF	Rabbit reticulocytes	Reverses inhibitory effect of HCR on ternary complex formation and on protein synthesis in reticulocyte lysates	370 000	[71]

initiation complex formation [26,27]. The stimulation is abolished by HCR and it has been proposed that phosphorylation of eIF-2 converts it to a form unable to interact with ESP [26,27]. Ranu and London have identified an eIF-2 stabilization factor (SF) which has similar properties to ESP and is required for HCR catalysed inhibition of ternary complex formation [28]. A similar activity to ESP has also been isolated from crude rat liver eIF-2 preparations [29]. This stimulates ternary complex formation and also has a GDPase activity which may be important in the control of eIF-2 activity. Dasgupta et al. have described yet another factor (CO-EIF 1, now called Co-eIF 2A) from reticulocytes which also stimulates ternary complex formation [30,31]. In addition, this group have isolated other proteins which act at the level of the ternary complex [32], one of which (TDI) may be identical to HCR [33,34]. Another (eIF-2B) now called CO-eIF 2C, has similar properties to ESP and SF [35].

It seems possible, therefore, that phosphorylation of eIF-2 by HCR prevents interaction of the factor with one or more co-factors, necessary for the normal functioning of eIF-2. A potential mechanism of action of such a co-factor could be the removal from eIF-2 of GDP, which is formed from GTP during initiation and is strongly inhibitory. Alternatively the rephosphorylation of eIF-2 bound GDP to GTP might occur so that a new ternary complex could then be formed. However, a recent report by Benne et al. [36] has cast some doubt on the authenticity of some co-factors which seem to stimulate ternary complex formation. This group has shown that, in assays measuring ternary complex formation in vitro, there are substantial losses of eIF-2 due to adsorption to the wall of the test tube, particularly at low concentrations. Addition of proteins isolated from lysates, while appearing to stimulate ternary complex formation, in fact only enhances recovery of eIF-2 and does not increase the activity of the factor. It may well be that some of the factors described above act in this way and therefore have little physiological significance.

Little is known about the mode of activation of HCR in the absence of haem and how the presence of haem prevents this. There is thought to be a conformational change involved in the conversion of proinhibitor to HCR with overt protein kinase activity [37]. Ochoa's group have proposed a mechanism for this activation based on the idea that the proinhibitor may be a substrate for a 3′,5′-cyclic AMP-dependent protein kinase [38–40]. (The HCR protein kinase activity itself is cyclic nucleotide independent.) Most of the evidence for Ochoa's model is quite indirect, however. Preparations of partially purified or apparently homogeneous protein kinase from bovine heart, or the catalytic subunit derived from it, were reported to inhibit protein synthesis in the reticulocyte lysate [38] and the isolated proinhibitor which is inactive in assays of protein synthesis or ternary complex formation, was rendered inhibitory by preincubation with the kinase or its catalytic subunit in an ATP-stimulated manner [38]. Haemin partially inhibited the activity of the cyclic AMP-dependent protein kinases from reticulocytes or bovine heart and prevented kinase-stimulated conversion of proinhibitor to HCR [39]. It also inhibited the binding of cyclic AMP to the protein kinase or its regulatory subunit and itself bound to these proteins [40]. Finally, cyclic AMP was claimed to stimulate formation of HCR from proinhibitor [39]. While this evidence appears to provide good support for Ochoa's model of HCR activation it has not been shown that the proinhibitor or HCR is a substrate for phosphorylation by exogenous protein kinase or that phosphorylation of any protein by this enzyme correlates with inhibition of protein synthesis. However, it is known that HCR is itself phosphorylated, perhaps autocatalytically, when incubated in vitro [11]. Proof of the model also requires it to be shown that inhibition of cyclic AMP-dependent protein kinases by haemin indirectly prevents phosphorylation of eIF-2. In contradiction to Ochoa's results, recent reports from two other laboratories in fact state that neither the reticulocyte cyclic AMP-dependent protein kinase nor cyclic AMP itself has any effect on protein synthesis in the reticulocyte lysate in the presence or absence of haem, and neither affects the phosphorylation of eIF-2 [41,42]. These results therefore cast considerable doubt on the validity of Ochoa's model.

A similar effect on protein synthesis in the reticulocyte lysate to that seen with haem deficiency is produced by the presence of low concentrations of double-stranded RNA (dsRNA) [43,44], or of oxidised glutathione (GSSG) [45] or on incubation at elevated temperatures [46]. As with haem deficiency, inhibition of protein synthesis in the first two situations is accompanied by activation of cyclic AMP-independent protein kinase activity that phosphorylates the small subunit of eIF-2 [45,47]. The inhibitory effects on

protein synthesis can be overcome by addition of exogenous eIF-2 [45,47]. The best characterised kinase, apart from HCR, is the dsRNA-activated protein kinase (dsI [47] or DAI [11]). This is antigenically distinct from HCR and is associated with the ribosomes rather than the post-ribosomal supernatant of lysates [47,48]. The mechanism of activation of this kinase appears to involve phosphorylation of a 67 000 mol. wt ribosome-associated polypeptide [47]. Paradoxically, high concentrations of dsRNA can reverse the inhibition of protein synthesis [17,49] and also prevent the phosphorylation of the 67 000 mol. wt protein [11].

dsRNA also activates another mechanism for inhibiting protein synthesis in the reticulocyte lysate which apparently does not involve protein kinases. dsRNA stimulates an enzyme which synthesises a series of small oligonucleotides, $pppA^{2'}p^{5'}A^{2'}p^{5'}A$ and longer oligomers (abbreviated here as 2–5A), from ATP [50]. These molecules in turn activate an endonuclease which degrades mRNA [51,52]. The relative contributions of the two types of mechanism to the inhibition of translation by dsRNA in the lysate depend on a number of factors, including the nature of the dsRNA, the KCl concentration and the type of mRNA being translated [53]. In general, low concentrations of naturally occurring dsRNA activate the protein kinase and inhibit initiation in a manner which is reversible by exogenous eIF-2 or high concentrations of dsRNA [53]. Intermediate concentrations of dsRNA, particularly synthetic polymers such as poly(rI):poly(rC), activate the 2–5A synthetase and endonuclease system; once the mRNA has been degraded, reversibility by eIF-2 is, of course, impossible [53]. Both types of translational control are also believed to be of considerable importance in the mechanism of action of interferon on protein synthesis in other cell types following virus infection.

3. Other systems controlled at the level of 40 S initiation complex formation

Although the regulation of initiation by haem in reticulocytes has been the most extensively studied system, there is now evidence that protein synthesis in other, less specialised cells is also controlled at the level of 40 S initiation complex formation.

Nutrient deprivation in intact Ehrlich ascites tumour cells reduces the rate of polypeptide chain initiation relative to that in fully fed control cells [54]. This is accompanied by a similar decrease in the level of [40 S · Met-$tRNA_f^{Met}$] complexes which can be observed both in vivo [55] and in extracts prepared from fed and starved cells [56]. The defect in 40 S complex formation can be overcome in extracts from amino acid-starved cells by exogenous eIF-2 [56]. It seems likely that starvation may activate an HCR-like protein kinase activity but this has not yet been shown. An inhibitor of 40 S initiation complex formation has been isolated from Ehrlich cells [57] but the role of this activity in the reduction of the rate of initiation of protein synthesis in nutrient deprivation remains to be determined.

The only hormonally sensitive system in which initiation complex formation has been assayed is the rat ventral prostate gland, which requires androgens for its normal growth and function. Castration of the male rat has been reported to decrease the ability of an eIF-2-like activity in the postribosomal fraction of the prostate to form ternary complexes with Met-$tRNA_f$ and GTP and to bind these to 40 S ribosomal subunits [58,59]. Whether this is due to the formation of a translational inhibitor is not known.

Resting lymphocytes exhibit a much reduced rate of polypeptide chain initiation compared to that observed in lymphocytes stimulated to proliferate by addition of mitogens [60]. Cell-free extracts from unstimulated cells also have a reduced ability to form 40 S initiation complexes compared to extracts prepared from phytohaemagglutinin-stimulated cells [61], and this difference can be overcome by addition of eIF-2 [62]. In this system, however, as well as 40 S initiation complex formation, later stages in the initiation pathway are known to be involved in the regulation of protein synthesis [61,62]. Lymphocytes also contain a translational inhibitor which appears to work by reducing the number of initiation complexes and is present in greater amounts in unstimulated cells [63], but whether this activity is responsible for inactivation of eIF-2 has yet to be determined.

The best characterised inhibitor of polypeptide chain initiation from non-erythroid cells is that which has been isolated from rat liver [64]. Its effect on protein synthesis in reticulocyte lysates, its chromatographic behaviour during isolation and its ability to phosphorylate the small subunit of eIF-2 suggest it is an HCR-like protein kinase. However, its physiological significance and its in vivo regulation are also still unknown.

Another example of control at the level of 40 S initiation complex formation is seen in cells treated with interferon. Part of the response to this agent involves the induction or activation of a latent protein kinase which, in the presence of dsRNA or after virus infection, phosphorylates eIF-2 [65,66]. It is not known if this enzyme is identical to the protein kinase activated by dsRNA in reticulocyte lysates, but it is tempting to speculate that the addition of dsRNA to reticulocyte cell-free systems mimics the effects of virus infection in interferon-treated cells.

4. Systems controlled at later states of initiation

Although most of the evidence to date suggests that initiation rates are controlled primarily at the level of 40 S initiation complex formation, there are indications that later stages in the initiation sequence may also be involved in regulation in some systems.

It has been claimed recently that the haem-controlled repressor in reticulocytes, while reducing the level of 40 S initiation complexes, also has an inhibitory effect on 80 S initiation complex formation [67]. This effect was independent of its effect on 40 S complexes and was thought to be due to inhibition of 60 S subunit joining. An accumulation of a 48 S initiation complex containing a 40 S subunit, mRNA and Met-tRNA$_f^{Met}$ was shown [67]. This finding has been contradicted by another group however [35]. Another situation where the level of 80 S initiation complexes is reduced is seen in cell-free extracts from unstimulated lymphocytes when compared to the level found in extracts from stimulated cells [61]. This difference may be overcome by the addition of a mixture of initiation factors but whether the effect is similar to that claimed for HCR in reticulocyte lysates is unclear [62]. A reduction in the level of 80 S initiation complexes in the presence of added mRNA is also seen in cell-free extracts from Krebs ascites cells heated to 45°C, compared to those incubated at 37°C [68]. This defect may be overcome by adding back a mixture of initiation factors.

It seems likely that control of the rate of polypeptide chain initiation is a more complex process than was once assumed. Modulation of several steps in the initiation sequence may be involved at the same time and situations are known to occur where several different control mechanisms act alongside one another to limit the rate of protein synthesis. An example of this is found in extracts from cells treated with interferon where the addition of dsRNA results not only in the activation of the protein kinase which phosphorylates the small subunit of eIF-2 [65,66], but also in the appearance of endoribonuclease activity which degrades mRNA [69,70]. It is likely that these two enzymes represent two distinct pathways for the inhibition of protein synthesis in interferon treated cell extracts; (their relative importance may depend on the conditions in the extract). These enzymes are also present in reticulocyte lysates, as mentioned earlier, and may therefore be part of a more general control mechanism limiting the rate of polypeptide synthesis.

5. Conclusions

This review has described the principal mechanisms by which the rate of polypeptide chain initiation is controlled in mammalian cells. Using the reticulocyte lysate as a model system it now appears likely that the control mechanisms found to operate here are also present in other cell types and may be of a more general nature than was once assumed. It also seems probable that more than one step in the initiation pathway is subjected to control in these systems and that several different control mechanisms may act alongside one another. An example of this is seen after interferon treatment and viral infection of cells in culture.

A greater understanding of events occurring during the regulation of polypeptide chain initiation in the reticulocyte lysate should come when the mechanism of inactivation of eIF-2 by HCR-catalysed phosphorylation is fully elucidated. While this appears to be imminent, any real understanding of the way in which haem deficiency activates HCR from the pro-inhibitor will require much more work. In non-erythroid cells effectors other than haem may also be involved in similar activations but almost nothing is yet known about the control of translational inhibitors which may be the analogues of HCR. Detailed insight into the mechanisms of HCR activation would therefore be a major advance from which such other information might be forthcoming.

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