Overview
Regulation of the rate of protein synthesis is of central importance in growth control. Treatment of quiescent cells with growth factors or hormones induces re-entry of cells into the cell cycle, accompanied by an increase in the synthesis of RNA and protein, and culminating in a doubling of protein mass before DNA synthesis and cell division [1]. At the basis of these controlling events are hormones, growth factors and mitogens that modulate the activities of specific target cells and are crucial components of the regulatory system that co-ordinates cell growth and development (reviewed in [2,3]). An increase in the rate of protein synthesis is obligatory for entry into, and progression through, the cell cycle, suggesting that activation of translation is an important event in the mitogenic response.

It is generally considered that the control of translation is exerted mainly at the level of polypeptide chain initiation. Simplistically, physiological regulation can be regarded as quantitative (i.e. affecting the overall rate) or qualitative (affecting translation of specific mRNAs); however, in many instances, both types of regulation may occur in concert, with larger rises in the translation of specific mRNAs superimposed upon a general increase in protein-synthesis rates. Apart from changes in the level of mRNA available for translation, regulation of the initiation phase of translation can be envisioned by two main routes: by modulating the efficiency with which an mRNA species interacts with the translational machinery, and by the activity of initiation factors that catalyse these interactions. The efficiency at which an mRNA is translated (or its 'translational strength') is dependent upon a number of features that influence its ability to compete for limiting initiation factors, which includes: the presence of the 5'-terminal cap, structural features of the mRNA at its 5' and 3' ends, and the consensus sequence flanking the initiator AUG codon (reviewed in [2–4]). In addition, the phosphorylation of initiation factors has a central role in modulating this process, possibly involving the co-ordinate phosphorylation of a number of components of the translational machinery [3].

eIF4E
All cytoplasmic mRNAs have a unique cap structure at their 5' terminus, which has a strong stimulatory effect on their translation; uncapped or non-methylated mRNAs are less competent at 48S and 80S initiation-complex formation than their capped counterparts [2–4]. Several cytoplasmic proteins can specifically interact with the mRNA cap structure, including four initiation factors, eIF4E, eIF4A, eIF4B and eIF4G (p220). Of these, only eIF4E specifically interacts directly with the cap structure, either as an individual polypeptide or part of a protein complex, termed eIF4F (Figure 1 and below). Mutagenesis experiments on the eIF4E protein have suggested that highly conserved tryptophan residues are important for interaction with the cap struc-

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Abbreviations used: MAPK or ERK, mitogen-activated protein kinase; NGF, nerve growth factor.
ture, with the sequence WEDE (amino acids 102–105 in human eIF4E) believed to be responsible for cap recognition. The protein has been isolated from a wide variety of sources, and the cDNAs corresponding to mRNAs encoding eIF4E have been cloned and sequenced from mammals, yeast, wheat and Drosophila [2-4]. Temperature-sensitive mutants of eIF4E and resultant eIF4E-depleted cell-free systems from yeast [5] have provided evidence for the importance of the factor. In the latter system, translation of most capped mRNAs was stimulated 5-7-fold upon addition of purified eIF4E, whereas translation of mRNA species with little secondary structure and cap requirement was only slightly stimulated. Disruption of the CDC33 gene in yeast, which encodes the eIF4E protein [5], causes a phenotype that is similar to that of a block at the G1-S border of the cell cycle, with the production of an eIF4E gene product with reduced affinity for the mRNA cap structure [5]. The primary structures of the human and mouse eIF4Es are almost identical but share only 33% similarity with the yeast protein. However, mammalian and yeast eIF4Es are functionally interchangeable in the eIF4E-dependent cell-free system [5].

Regulation of eIF4E activity
As possibly one of the first initiation factors to interact with the mRNA, eIF4E would seem to be an ideal target for regulation of translation initiation. This may be mediated by phosphorylation, which is discussed in detail below, or by modulating the level of eIF4E in the cell available to participate in the initiation process. Recent evidence has indicated that these processes of regulation are distinct, independent mechanisms for controlling cap-dependent protein synthesis that can act either in concert or in opposition to each other [6].

Regulation of availability of eIF4E
Cellular concentrations of eIF4E have been estimated to be low relative to those of other components of the translational machinery, and eIF4E is widely regarded as potentially rate limiting in amount [2-4], although for the reticulocyte lysate, this has recently been challenged [7]. Genetic manipulation strategies that can alter the intracellular levels of eIF4E have shown that overexpression of eIF4E can exert profound effects on cell growth, resulting in aberrant mor-
was apparent at later times of cell activation [19]. Elevated levels of eIF4E mRNA and protein have also been observed in a variety of transformed cell lines and tumours [20,21], although it is unclear whether this is simply a consequence of a general increase in the translational apparatus. More recently, a physiological mechanism for regulating the availability of eIF4E on an acute basis has been determined. Pause et al. [22] and Lin et al. [23] screened a placental cDNA expression library to identify proteins that interact with eIF4E. In addition to eIF4G and three other proteins that have not been characterized, the screen identified two proteins (4E-BP1 and 4E-BP2) that have 56% sequence identity at the amino acid level. 4E-BP1 was found to be 93% identical to PHAS-I, which was previously identified as a protein rapidly phosphorylated in adipose tissue in response to insulin [16,24]. Interaction of 4E-BP1 with eIF4E inhibits cap-dependent translation both in vitro and when the protein is expressed in cells, 4E-BP1 does not interfere with the cap recognition by eIF4E, but prevents the assembly of eIF4E into the eIF4F complex [2-4,7,25]; indeed, a sequence similarity has been identified between a region of 4E-BP1 and the eIF4E-binding site on eIF4G. Treatment of adipocytes with insulin caused 4E-BP1 to become hyperphosphorylated and dissociate from eIF4E, thereby relieving the translational inhibition (see Figure 1). Initially this phosphorylation was proposed to be mediated via the mitogen-activated protein kinase (MAPK; ERK) signalling pathway, which could directly phosphorylate 4E-BP1 in vitro. However, numerous studies have now shown that 4E-BP1 can be phosphorylated in the absence of activation of the MAPK pathway, suggesting that other kinases may be involved in the dissociation of the eIF4E/4E-BP1 complex [25-27]. As shown in Figure 1, these may include casein kinase II, protein kinase C, protein kinase A and p70S6K. Studies using rapamycin, a macrolide immunosuppressant that prevents the activation of p70S6K S6 kinase [28], have implicated this pathway in mediating the phosphorylation of 4E-BP1 in mitogen-stimulated cells [29-35], although the physiological kinase(s) that influence the association of 4E-BP1 with eIF4E are yet to be characterized.

Phosphorylation of eIF4E

The phosphorylation of eIF4E in many cell systems is increased in response to agents that stimulate cell growth, including growth factors, hormones, phorbol esters and phosphatase inhibitors, as well as the expression of oncogenic signalling molecules (reviewed in [2,3]). It is widely assumed that such changes in the phosphorylation of eIF4E mediate changes in both the overall rate of protein synthesis and the pattern of recruitment of individual mRNAs for translation, although the exact molecular mechanism awaits determination. Studies utilizing m7-GTP-Sepharose affinity resins have shown that phosphorylation of eIF4E alone is not a pre-requisite for its interaction with the mRNA cap structure (reviewed in [2-4,7]). However, more subtle experiments have shown that the phosphorylated form of eIF4E binds 3-4-fold more tightly to capped oligonucleotides than does the non-phosphorylated form [36]. In a more physiological assay system, luciferase mRNA constructs (either with or without the cap structure) were electroporated into 3T3-L1 cells; in response to either insulin or serum, a strongly cap-dependent stimulation of translation of these reporter constructs was demonstrated under conditions where the level of eIF4E phosphorylation was increased [37]. In the case of complex formation with eIF4G and other initiation factors, there have been several observations in intact cells of parallel changes in the phosphorylation state of eIF4E and its recruitment into high-molecular-mass complexes [38-44]. Isolation of ribosome-associated eIF4E shows that this population also contains a large proportion of unphosphorylated protein [7], indicating that, in the reticulocyte lysate, phosphorylation of eIF4E is neither necessary nor sufficient for involvement of the factor in initiation complexes.

Until recently it had been believed that eIF4E was phosphorylated on a single serine residue, Ser-53 (reviewed in [2-4,45]). However, transient transfection studies in COS cells by Kaufman et al. [46] clearly showed that not only was the Ala-53 variant phosphorylated to the same extent as the wild-type protein, but also that it was recruited into the eIF4F complex with the same efficiency as the Ser-53 protein. These data showed that phosphorylation of eIF4E at Ser-53 was not required for eIF4F complex formation or efficient cap-dependent translation in COS cells. It was also suggested that the possession of an alanine or glutamic acid residue at position 53 somehow alters the characteristics of eIF4E in some way relative to the Ser-53-containing protein, without affecting recognition of the mRNA cap structure, as all variants interact
with the cap structure [45]. These findings are somewhat similar to the confusion seen with Ser-to-Ala mutants of another initiation factor, eIF2α [3,47]. As a result, the phosphorylation site on eIF4E has been reappraised and is now determined to be Ser-209 in reticulocyte lysates [48], in CHO.T cells stimulated with serum [49], or following phosphorylation with protein kinase C in vitro [50]. Studies carried out with recombinant proteins have suggested that, in addition to Ser-209, phosphorylation on Thr-210 may also occur during in vitro phosphorylation of eIF4E [50,51]. It is interesting to note that an equivalent site to Ser-209 is present in Schizosaccharomyces pombe (P. Curtis and S. Morley, unpublished work) but absent from the Drosophila, Saccharomyces cerevisiae and one form of wheat eIF4E, raising questions as to whether phosphorylation of eIF4E really has a universal role in the control of cell growth (sequences compared in [52]).

As depicted in Figure 2, the identity of the physiological eIF4E kinase(s) presently remains unclear. eIF4E is phosphorylated in vivo in response to the phorbol ester PMA in reticulocytes, 3T3-L1 cells or T-cells [40–44,53], and in vitro by protein kinase C [50,54]. Furthermore, down-regulation of protein kinase C by prolonged exposure to phorbol ester blocks both the PMA- and the insulin-induced increase in eIF4E phosphorylation in 3T3-L1 cells. Microinjection of eIF4E or eIF4F (but not other initiation factors) will activate DNA synthesis in serum-starved NIH 3T3 cells, an effect that is potentiated by co-microinjection of protein kinase C [13]. However, it appears that both protein kinase C-dependent and -independent pathways are involved in phosphorylation of eIF4E, depending upon the stimulus and cell type in question [1–4]; the nerve growth factor (NGF)-induced response in PC-12 cells is unaffected by protein kinase C down-regulation [55], and the enhanced phosphorylation of eIF4E seen in lipopolysaccharide-stimulated B-lymphocytes is insensitive to the protein kinase inhibitors H7 and HA 1004, which are inhibitors (albeit not very specific ones) of protein kinases C and A [56].

During the stimulation of cell growth, multiple independent, converging signalling pathways are activated to relay signals from the membrane to the translational machinery, which includes eIF4E (Figure 2). Enhanced phosphorylation of eIF4E in response to serum or insulin is not prevented by rapamycin, whereas the phosphorylation of 4E-BP1 is blocked by this drug.

**Figure 2**

Proposed signalling pathways regulating the enhanced phosphorylation of eIF4E

Abbreviations used: GCK, germinal-centre kinase; LPS, lipopolysaccharide; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKB, protein kinase B; mTOR, mammalian target of rapamycin; MEK, MAP kinase; MKK 3/6, MAP kinase kinase 3/6; KsrI, MEK2 kinase; PAK1, protease-activated kinase 1; MEKK1, MAP kinase kinase 1; SEK1, stress-activated protein kinase kinase; SAPK, stress-activated protein kinase; MAPK APK2, MAPK-activated protein kinase 2; 4E P, phosphorylated eIF4E.
These studies indicate that the activity of p70^S6K is not required for enhanced phosphorylation of eIF4E, but activation of this pathway is required for dissociation of the eIF4E-4E-BP-1 complex, which may influence the rate of phosphorylation of eIF4E [50].

There is evidence that ras can regulate the phosphorylation of eIF4E [11]. In PC-12 cells, expression of a trans-dominant negative form of ras that abrogates function prevents both cellular differentiation and NGF-induced eIF4E phosphorylation [55]. However, as Ras itself does not possess intrinsic protein kinase activity, at least one serine/threonine kinase must link Ras to eIF4E. One possible link to eIF4E is the MAP kinase signalling pathway and related families of enzymes (Figure 2; [58,59]). By using the MAPK kinase-specific kinase inhibitor PD98059, Flynn and Proud [60] have shown that the insulin-stimulated phosphorylation of eIF4E in CHO.T cells is mediated by the classical MAP kinase pathway. Similar results have been obtained with PD98059 in serum-stimulated NIH 3T3 cells and Xenopus kidney cells in culture; in these cells, PD98059 did not influence eIF4E association with 4E-BP1, and the inhibition of phosphorylation of eIF4E in these instances was not complete (Figure 2; C. Fraser and S. Morley, unpublished work), possibly reflecting signalling through Ras1 and MAPK kinase-2 [58]. In vitro studies have shown that c-Raf, MAP kinases and p90^RS will not directly phosphorylate eIF4E [1–3]. Studies with Xenopus oocytes have suggested that eIF4E phosphorylation is increased at a time when the MAP kinase signalling pathway is activated during meiotic maturation or upon microinjection of Ras protein [44]. Activation of the stress-activated protein kinases also leads to the enhanced phosphorylation of eIF4E. Treatment of NIH 3T3 cells with anisomycin led to phosphorylation of eIF4E, an effect that was additive to that seen with serum alone. It is likely that this enhanced phosphorylation was mediated via activation of mTOR/p70^S6K pathway and the p38 pathway (Figure 2), as it was prevented by rapamycin and by SB203580 (S. Morley, unpublished work). Following heat shock of Xenopus kidney cells, there is also an enhanced phosphorylation of eIF4E that is unaffected by PD98059 but completely abrogated by SB203580 (C. Fraser and S. Morley, unpublished work).

It is quite possible that these kinases are upstream regulators of as-yet-unidentified initiation-factor protein kinases, which are also part of super-families of enzymes (Figure 2). Evidence for such a suggestion comes from the finding that eIF4E can be phosphorylated in vitro at the physiological site (Ser-209) by a bovine kidney protamine kinase [61], which is in turn activated in a hormone-sensitive manner by kinases with properties similar to but distinct from MAP kinases [62]. It is also important to appreciate that the regulation of eIF4E phosphorylation involves modulation of both kinase and phosphatase activities, possibly reflecting increased turnover of phosphate on eIF4E following stimulation of cell growth. This has been shown in B-lymphocytes [56], NGF-stimulated PC-12 cells [55], src-transformed NIH 3T3 cells [63] and ras-transformed rat-embryo fibroblasts [64]. This suggests the possibility that it is not the phosphorylation of eIF4E that is important for function, but rather the phosphate turnover between successive rounds of initiation that plays a central role in the regulation of this factor [3]. Although protein phosphatase 2A has been demonstrated to dephosphorylate eIF4E in vitro [51,65], the physiological significance of this observation is unknown. Therefore, identification of these kinase(s) and/or phosphatase(s) and characterization of the signalling pathways that modulate the phosphorylation of eIF4E are of paramount importance to our understanding of the regulation of cell growth.
Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation

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Introduction

The double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is a 68 kDa (human) or 65 kDa (mouse) serine/threonine kinase that is induced by interferon (IFN) (reviewed in [1]). PKR regulates protein synthesis by phosphorylating the α subunit of initiation factor eIF2, resulting in sequestration of limiting pools of eIF2B and rapid inhibition of translation. PKR is normally inactive, but on binding dsRNA it undergoes a conformational change allowing autophosphorylation and dsRNA-independent phosphorylation of substrates. Other polyanionic molecules, such as heparin, dextran sulphate, chondroitin sulphate and poly(L-glutamine) are also able to activate PKR in vitro, although the binding site(s) for heparin appear to be different from those regions identified as binding dsRNA [2]. In cell culture, PKR can be activated by exposure to calcium ionophores [3,4] or stress inducers such as sodium arsenite or lipopolysaccharides [5,6]. Cytokines, including IFNs α/β, γ, interleukin 3 and tumour necrosis factor (TNF), also activate PKR, and growth factors such as platelet-derived growth factor may also signal through PKR-dependent pathways [7-12].

Whether any of these agents requires a dsRNA intermediate remains to be determined.

PKR characteristics

Details of the interaction of dsRNA with PKR have been derived from biochemical and mutational analyses and by comparison with structural information from NMR spectroscopy studies of related dsRNA-binding proteins (reviewed in [13]). The conserved dsRNA-binding motifs of PKR are located at amino acids 55–75 and repeated at 145–166 of the 551-amino-acid human kinase, but the first domain alone is sufficient to mediate dsRNA-binding activity. Amino acids critical for dsRNA binding are highly conserved among dsRNA-binding proteins. Positively charged lysine residues are predicted to form ionic bonds with the negatively charged phosphodiester backbone, and other residues may stabilize secondary structure. There is no sequence specificity involved in dsRNA binding to PKR, and the importance of single/basic residues suggests that ionic interactions between positively charged amino acids and the negatively charged phosphodiester backbone contribute to the RNA-binding mechanism.

Since the binding of dsRNA is required to unmask the ATP-binding site, it seems probable that the N-terminus of PKR is in contact with the C-terminus. Accordingly, the binding of dsRNA to PKR causes a conformational shift, as measured by tryptophan fluorescence [14], that presumably unmaskes the C-terminus. Small-