Natural occurrence and physiological role of a truncated eIF4E in the porcine endometrium during implantation

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The present study is the first report providing evidence for a physiological role of a truncated form of the mRNA cap-binding protein eIF4E1 (eukaryotic initiation factor 4E1). Our initial observation was that eIF4E, which mediates the mRNA cap function by recruiting the eIF4F complex (composed of eIF4E, 4G and 4A), occurs in two forms in porcine endometrial tissue in a strictly temporally restricted fashion. The ubiquitous prototypic 25 kDa form of eIF4E was found in ovariectomized and cyclic animals. A new stable 23 kDa variant, however, is predominant during early pregnancy at the time of implantation. Northern blotting, cDNA sequence analysis, in vitro protease assays and MS showed that the 23 kDa form does not belong to a new class of eIF4E proteins. It represents a proteolytically processed variant of eIF4E1, lacking not more than 21 amino acids at the N-terminus. Steroid replacements indicated that progesterone in combination with 17β-oestradiol induced the formation of the 23 kDa eIF4E. Modified cell-free translation systems mimicking the situation in the endometrium revealed that, besides eIF4E, eIF4G was also truncated, but not eIF4A or PABP [poly(A)-binding protein]. The 23 kDa form of eIF4E reduced the repressive function of 4E-BP1 (eIF4E-binding protein 1) and the truncated eIF4G lacked the PABP-binding site. Thus we suggest that the truncated eIF4E provides an alternative regulation mechanism by an altered dynamic of eIF4E/eIF4G binding under conditions where 4E-BP1 is hypophosphorylated. Together with the impaired eIF4G–PABP interaction, the modified translational initiation might particularly regulate protein synthesis during conceptus attachment at the time of implantation.

Key words: implantation, oestradiol, progesterone, porcine endometrium, translation initiation, truncated eukaryotic initiation factor 4E1 (eIF4E).

INTRODUCTION

Protein synthesis is a major determinant of cell growth and differentiation. In the context of conceptus implantation, maternal ovarian steroids and embryonic signals, such as oestrogens [1,2], have been found to influence proliferation and differentiation of the endometrium by modulating those signalling cascades regulating mRNA translation. This view is supported by results showing that signal transduction pathways involving the MAPKs (mitogen-activated protein kinases) and the serine/threonine protein kinase Akt can be activated by 17β-oestradiol [3,4] or P4 (progesterone) [5]. Among other processes, these kinases modulate protein synthesis at the translational level [6–8]. The main targets are translational initiation factors [eIFs (eukaryotic initiation factors)] such as the mRNA-cap-binding protein eIF4E and its repressors 4E-BP1 and 4E-BP2 (eIF4E-binding proteins 1 and 2).

In eukaryotes, more than 95% of proteins are synthesized via cap-dependent translation. In this context, eIF4E plays a key role in the recruitment of mRNAs to ribosomes [9]. The first detected prototypical eIF4E is found in all eukaryotes. It has an apparent molecular mass of approx. 25 kDa and might be the rate-limiting factor in translational initiation [10,11]. This process is influenced by the secondary and tertiary structure of the 5′-UTR (5′-untranslated region) of the mRNA and by the cap structure (m7GpppN) of the mRNA molecules. The functional cap-binding complex eIF4F is composed of the mRNA cap-binding protein eIF4E, eIF4A, an RNA helicase responsible for the unwinding of secondary structures of mRNAs, and eIF4G serving as a scaffold protein for the assembly of the complex [11]. eIF4F mediates the cap function during translation initiation. However, today the most sophisticated model of translational stimulation is the so-called ‘closed loop’ model [12,13]. Simultaneous interactions between the 5′-cap and the 3′-poly(A) sequence of the mRNA are mediated by the protein factors, whereby eIF4G provides binding sites for eIF4E, eIF4A, eIF3 (which bridges eIF4E to the small ribosomal subunit), PABP [poly(A)-binding protein], MNK (MAPK-interacting kinase), the kinase which directly phosphorylates eIF4E, and also for RNA. This complex facilitates ribosome binding and stimulates translation efficiency 8–10-fold [14].

The biological activity of the cap-binding protein eIF4E can be regulated at different levels. First, eIF4E is phosphorylated at Ser209 in response to a variety of stimuli such as growth factors, cytokines or amino acids [15]. It was shown that eIF4E phosphorylation reduces its affinity to the cap structure. eIF4E

Abbreviations used: ACTB, actin β-chain; C10 etc., day 10 of the oestrous cycle etc.; EB, oestradiol benzoate; eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; GST, glutathione transferase; MALDI–TOF-TOF, matrix-assisted laser-desorption ionization–time-of-flight–time-of-flight; MAPK, mitogen-activated protein kinase; MNK, MAPK-interacting kinase; Nt-NTA, Nt2- nitritotriacetate; OVX, ovariectomized/ovarioectomy; P4, progesterone; P13 etc., day 13 of pregnancy etc.; PABP, poly(A)-binding protein; RRL, rabbit reticulocyte lysate; SPR, surface plasmon resonance; 5′-UTR, 5′-untranslated region.

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release from the cap can probably bind to a different subset of mRNAs and enhance the translation rate [16].

Secondly, eIF4E-binding proteins can work as repressors of eIF4E function. Specific proteins called 4E-BP1, 4E-BP2 and 4E-BP3 were shown to act as competitors of eIF4E/4G binding. When hypophosphorylated, they form complexes with eIF4E and prevent the formation of the functional eIF4E complex [17]. 4E-BP1 and 4E-BP2 have at least six potential phosphorylation sites, and hierarchical phosphorylation reduces their affinity to eIF4E as well as suppressing their inhibitory effect on the translational initiation [7,18].

Thirdly, subclasses of eIF4E encoded by different genes (eIF4E classes 1–3) or splicing variants have been detected in many organisms from different species [19,20]. Generally, some eIF4E-related proteins may act as translation factors and stimulate either global mRNA recruitment or the recruitment of a subset of mRNAs. Others may exhibit only partial activity and thus act as inhibitors of translation. However, the complexity of the biological function of the different members of the eIF4E family is not fully understood and remains to be investigated in detail, especially in the light of recently detected new eIF4E-binding partners [20].

Proteolytic processing resulting in stable variants has never been detected for eIF4E in a physiological context, in contrast with other initiation factors such as eIF4G [21] or PABP [14]. However, structural analyses with recombinant N-terminally truncated mammalian eIF4E forms have been performed [22,23]. The N-terminal region of eIF4E, from amino acids 1 up to Gln40, appeared to form a unique flexible unstructured segment. It was suggested that this segment is dispensable for cap-dependent translation. A truncation of 33 amino acids from the N-terminus yielded a protein binding to the cap structure with the same affinity as the full-length eIF4E [22]. More recently, however, a regulatory impact of the N-terminal segment was suggested [24]. Deletions in this area result in different binding of the repressors 4E-BP1 and 4E-BP2. These alterations depend on the length of the deleted segment and on the interaction state of both molecules [23–25].

In respect to eIF4G binding, deletions of up to 20 amino acids from the N-terminus of eIF4E from yeast did not result in a reduced affinity to eIF4G compared with the wild-type. In contrast, deletions of larger segments (up to 35 amino acids) result in a significantly reduced affinity [26].

In the present study, we analysed the expression of eIF4E in the porcine endometrium during implantation. At this time, as a consequence of embryonic/maternal cross-talk, structural and functional alterations occur in the endometrial epithelium, allowing attachment of blastocysts and their further development [27]. Surprisingly, we found a naturally occurring proteolytically processed eIF4E variant. The truncated 23 kDa form persists as a stable variant in the porcine endometrium during implantation. Taken together, our results suggest that the proteolytic cleavage of eIF4E represents an alternative regulatory mechanism of translational initiation, which is distinct from the well-known regulation through phosphorylation of eIFs. An altered dynamic of eIF4E/4E-BP1 binding, together with the impaired eIF4E–PABP interaction also observed, might be important in regulating endometrial protein synthesis, particularly during non-invasive implantation in pigs.

EXPERIMENTAL

Details of the materials and methods used for RNA analysis, Northern blotting and cDNA analysis are available in the Supplementary Experimental section at http://www.BiochemJ.org/bj/432/bj4320353add.htm.

Animals, steroid replacements and unilateral pregnant uterine horn

For all of the animal studies, ethical guidelines were followed and the research has been authorized by German authorities (VI-522a-7221.31-1-036/00).

Mature German Landrace gilts (Sus scrofa domesticus; 8.5 months of age, 120–125 kg) were oestrus- and ovulation-synchronized as described previously [28]. Gilts were hysterecomitized on C13 [day 13 of the oestrus cycle (n = 7) and on P13 [day 13 of pregnancy (n = 10)], P15 (n = 15; including five animals used for the model unilateral pregnant uterine horn; see below) and P30 (n = 7) [gilts were bred by artificial insemination 24 and 38 h after hCG (human chorionic gonadotropin)]. Additionally, on C10, 24 gilts were OvX (ovariectomized). Steroid replacements with EB (oestradiol benzoate) and P4 were performed as described previously [28]. In further experiments, one uterine horn from each of five pigs was endoscopically closed with a staple line of titanium clips before insemination. This treatment results in pregnancy in one uterine horn only.

Preparation of endometrial tissue extracts

For analysis of initiation factors, 1.5 g of endometrial tissue was used and the homogenate was prepared in the presence of protease inhibitors as described previously [28], except that the EDTA concentration was 10 mM. Supernatants were used for immunoblots and protein–protein interaction assays. For in vitro protease assays, the tissue was extracted with a buffer containing 150 mM NaCl and 20 mM Tris/HCl (pH 7.2) without any protease inhibitors. The protein concentration was determined as described previously [29]. Granulosa cells, in vitro-matured porcine oocytes and blastocysts were handled as described previously [30,31]. Furthermore, tissues from other organs (muscle, heart, kidney, brain, ovary, spleen and liver) were homogenized as described above.

Protein analysis

For the analysis of eIF4F components, PABP, 4E-BP1 and 4E-BP2 by Western blotting, the standard SDS/PAGE and transfer method was used [32]. The blots were developed with the ECL-Plus detection system and were digitalized using the Camilla Camera System (raytest). The absorbance of the bands was measured and evaluated by Aida software (raytest). Binding assays with the different eIF4E forms to m7-GTP–Sepharose was performed as described previously [31]. For the in vitro protease assay, 50 ng of N-terminally GST (glutathione transferase)-tagged full-length eIF4E (51 kDa) served as an external substrate to be cleaved by endometrial lysates (20 μl) collected after OvX and on P15. Incubation was performed for 0, 30, 60 or 120 min at 37°C. In parallel assays, the samples were treated with 5 mM EDTA or EGTA supplemented with or without 10 mM CaCl2. The reaction was stopped by adding 5× concentrated SDS/PAGE sample buffer. Samples were analysed by Western blotting and were probed for eIF4E and GST respectively. For semi-preparative eIF4E cleavage and subsequent MS analysis, 1 μg of GST–eIF4E fusion protein was used as an external substrate. To avoid high contamination with endometrial proteins and to achieve maximal cleavage, the cleavage reaction was carried out sequentially by serial addition of OvX or P15 endometrial lysates, diluted 1:20, for 30 min at 37°C. The addition of diluted lysate was repeated four times. The cleavage reaction was monitored on
Coamassie-Blue-stained gels after SDS/PAGE and by Western blotting. For MS analysis, tryptic in-gel digestion was performed and the MALDI–TOF–TOF (matrix-assisted laser-desorption ionization–time-of-flight-time-of-flight) measurement was carried out on the 4800 MALDI–TOF–TOF analyser (Applied Biosystems) as described previously [33]. For the database search, the Mascot search engine (version 2.104; Matrix Science) with a specific NCBI (National Center for Biotechnology Information) sequence database was used.

**In vitro translation and protein–protein interaction analysis**

For *in vitro* translation, nuclease-treated RRL (rabbit reticulocyte lysate) from Promega was used to synthesize biotinylated proteins. To evaluate the elf4E dependency of translation or to mimic the situation in the endometrium, the RRL was modified as follows. In the first case, elf4E was depleted from the RRL by m7-GTP–Sepharose as described previously [34] and the RRL was subsequently supplemented either with recombinant elf4E (0.75 μl/50 μl of assay volume) or unphosphorylated His-tagged 4E-BP1 (0.75 μl/50 μl of assay volume). In the second case, the complete RRL was subjected to limited proteolysis by supplementation with endometrial lysates obtained after OVX or on P15. Titration studies were carried out in advance to achieve optimal cleavage of elf4E and elf4G while avoiding exorbitant contamination of the RRLs with endometrial proteins. Lysates containing 1.5 μg of protein were added to 50 μl of RRL. In additional assays, the treated RRLs were supplemented with recombinant 4E-BP1 (1 μg/50 μl of RRL). All treatments of the RRLs were monitored by Western blotting. The *in vitro* translation reactions were carried out in a final volume of 50 μl with 60% RRL, 140 mM potassium acetate, 0.5 mM magnesium acetate, 10 mM calcium phosphate, 1 mM amino acid mix and 1 μl of Transcend™ tRNA. The RNA was stabilized by addition of 1 μg of RNasin. The reaction was started by adding 50 ng of capped polyadenylated Xef1 mRNA. Samples were taken at zero time, and after 15 and 30 min. Biotinylated Xef1 translation products were analysed by Western blotting and visualized by streptavidin–HRP (horseradish peroxidase) detection as described previously [35]. Directly after the *in vitro* translation reaction, the RRLs were analysed by immunoprecipitation with immobilized anti-elf4E as described previously [32] and probed for elf4E, elf4G, elf4A, PABP1 and 4E-BP1. To analyse the binding of the elf4E variants to 4E-BP1, recombinant N-terminally His-tagged full-length 4E-BP1 was bound to Ni-NTA (Ni²⁺-nitrioltriacetate) magnetic agarose beads according to the manufacturer’s instructions. His-tagged 4E-BP1 (15 μl of slurry; 10 μg of His-tagged 4E-BP1) bound to the beads was incubated with endometrial lysates after OVX and on P15 for 2 h at 4°C under constant agitation. As negative controls, lysates were incubated with the beads and without His-tagged 4E-BP1 under the same conditions. The beads were washed three times with buffer containing 50 mM NaH₂PO₄, (pH 8.0), 150 mM NaCl, 20 mM imidazole and 0.005 % Tween 20. Proteins were eluted from the resin with a buffer of the same composition supplemented with 250 mM imidazole. Bound fractions and precipitated supernatants were analysed by Western blotting and probed for elf4E and His respectively.

**RESULTS AND DISCUSSION**

**A 23 kDa elf4E form is preferentially expressed in the endometrium during implantation**

A key result of our present study is the detection of a 23 kDa small-size variant of the mRNA-cap-binding protein elf4E in the porcine endometrium during early pregnancy at the time of implantation.

Western blot analysis for elf4E (Figure 1A) in the endometrial cytosol of pigs after OVX, on C13 and on P13, P15 and P30 revealed a single band with a molecular mass of 25 kDa in the endometrium after OVX. The same band was predominantly detected on C13. On P13 and P15, the 25 kDa band disappeared almost completely and a band of approx. 23 kDa emerged. Both bands appeared on P30. A similar pattern was obtained when a phospho-specific antibody was used. The abundance of the truncated elf4E significantly increased on P15 (Figure 1A).

To demonstrate that the elf4E cleavage is not an artifact of the extract preparation, samples after OVX and on P15 were homogenized either separately as controls or after mixing both tissues. Clearly, extraction of the mixed samples did not reduce the abundance of the prototypical 25 kDa elf4E nor did additional cleavage products appear (Figure 1B). This indicates that the elf4E truncation in P15 samples is not an extraction artifact.

Analysis of various other porcine organs (muscle, heart, kidney, brain, ovary, spleen and liver) and cells (oocytes, blastocysts, fibroblasts and granulosa cells) did not reveal a truncated form of elf4E (Figure 1C). However, traces of smaller proteins were found in the kidney and liver samples. They differ in size compared with the endometrial 23 kDa elf4E form and could not be detected with the phospho-specific antibody. We conclude that these proteins are not identical with the endometrial 23 kDa elf4E (see below) and they were not investigated further. Additionally, both the 23 and 25 kDa elf4E could be precipitated by m7-GTP–Sepharose (Figure 1D).

Further investigations revealed that, in the endometrial lysates on P15, elf4G was affected by extensive proteolytic cleavage generating discrete fragments (Figure 1E). However, other factors such as elf4A and PABF were not cleaved. Additionally, this assay revealed a reduced abundance of 4E-BP1 on P15. Moreover, 4E-BP1 appeared in the α-band, which has been shown previously to be the hypophosphorylated form [31]. In contrast, the abundance of 4E-BP2 was not significantly reduced on P15, but a shift to a higher electrophoretic mobility was also observed. We speculate that 4E-BP2 is dephosphorylated on P15 as well. However, the phosphorylation of this protein has not been as studied extensively, thus we cannot exclude that the observed faster migration of 4E-BP2 could even result from truncation. Nevertheless, these different events, namely elf4G fragmentation, 4E-BP1 dephosphorylation and partial degradation and 4E-BP2 dephosphorylation/truncation, suggest that these modifications are regulated processes specific during early pregnancy.

We envisage that the generation of the truncated elf4E is a regulated process as well. elf4E and elf4G fragments are stably expressed and not an intermediate product arising through general degradation. No signs of apoptosis or general proteolysis were observed in P15 samples. For example, no difference in active caspase 3 after OVX or on P15 was detected. Moreover, cultures of endometrial luminal epithelial cells from P15 remained viable. However, the 23 kDa elf4E gradually disappeared in these cells and was replaced by the 25 kDa form after at least 24 h (results not shown).

The truncated elf4E is a proteolytically processed product of the prototypical 25 kDa elf4E protein

Northern blotting and cDNA sequence analysis showed no indication for the occurrence of an alternatively spliced mRNA or an alternative gene encoding the truncated factor (see Supplementary Figures S1 and S2 at http://www.BiochemJ.org/
Figure 1 Expression of eIF4E in the porcine endometrium and various other organs and cells, and analysis of eIF4G, eIF4A, PABP, 4E-BP1 and 4E-BP2 on P15

Expression and phosphorylation of eIF4E. (A) Two biological replicates from endometrium after OVX, C13 and P13, P15, P30 are shown. The blots were reprobed for ACTB (actin β-chain) as a loading control. The bar charts show the 25 kDa eIF4E (black) and the 23 kDa form (white) in relation to the abundance of the loading control ACTB (OVX: n = 6; C13: n = 7; P13: n = 10; P15: n = 15; P30: n = 7). Values are means ± S.E.M. *P < 0.05 between the abundance of the 25 kDa and 23 kDa forms of eIF4E. (B) Extracts from OVX and P15 tissue were homogenized in protease-inhibitory conditions, alone or together (mix) as indicated. (C) The expression of eIF4E in various porcine tissues and cells. (D) The ability of both eIF4E forms to bind to m7-GTP–Sepharose. C is the control after OVX; the input (in) and the bound fraction (b) after OVX from cyclic gilts (C13) and from P13 and P30 are shown. (E) Analysis of the expression of eIF4G, eIF4A, PABP, 4E-BP1 and 4E-BP2 in the endometrium on P15 compared with the situation after OVX. Representative blots from three biological replicates are shown in (C–E).

Therefore we analysed whether the 23 kDa eIF4E was generated from the 25 kDa form by proteolytic cleavage (Figure 2). The in vitro protease assay with a GST–eIF4E fusion protein (51 kDa) as an external substrate revealed that the cell lysate after OVX did not generate any cleaved eIF4E, even when Ca2+ was added. In contrast, the P15 lysate generated a distinct fragment of ∼23 kDa (Figure 2A). Here, the amount of the recombinant eIF4E decreased with the duration of incubation, while the truncated form increased. This reflects the sum of the 23 kDa form present in the P15 lysate and the newly processed 23 kDa eIF4E generated from the recombinant external substrate. Additionally, the GST blot showed an ∼28 kDa fragment which must be composed of the 26 kDa GST tag linked to an approx. 2 kDa N-terminal eIF4E fragment. The results also revealed that the eIF4E cleavage was strongly inhibited by EDTA which can be revoked by applying additional CaCl2, suggesting the action of a Ca2+-dependent protease. In an additional experiment, the more Ca2+-specific chelator EGTA was used to analyse eIF4E cleavage (Figure 2B). The results showed an identical effect as that obtained with EDTA treatment. Quantification of the GST blot is presented in Figure 2(C).

In general, maximal cleavage was already reached after 30 min of incubation. The truncated eIF4E remained stable for at least 20 h. No further degradation of the truncated eIF4E was observed, even after that long duration of incubation (results not shown).

Lysates from kidney or liver cells, which also had traces of a fast-migrating band (Figure 1C), could not cleave the GST-tagged eIF4E (results not shown). These results support the notion that...
Figure 2  In vitro protease assay with recombinant GST–eIF4E fusion protein as an external substrate in endometrial lysates after O VX and on P15

(A) Endometrial extracts after O VX and on P15 and the external substrate were incubated for 0, 0.5, 1 and 2 h, in the presence of EDTA/CaCl₂ and EDTA alone as indicated. Western blots of the assays for eIF4E (upper panels) and GST (lower panels) after O VX (left-hand panel) and on P15 (right-hand panel) are shown. Controls (C) were cell extracts from porcine liver (eIF4E blot after O VX) and GST-tagged eIF4E protein (GST blot after O VX); and cell extracts from porcine liver (eIF4E blot on P15) and GST protein (GST blot on P15). (B) Comparison of the effect of EDTA and EGTA on eIF4E–GST cleavage in endometrial P15 samples. Samples were substituted with EDTA, EGTA and CaCl₂ as indicated and incubated for 90 min or not incubated (0) as a control. The Western blots for eIF4E and GST are shown. (C) Quantification of the GST blots from (B).

these proteins are not identical with the endometrial 23 kDa form of eIF4E.

Generation of discrete fragments through proteolytic cleavage has been reported to affect different initiation factors in distinct physiological situations [21]. For instance, eIF4G, eIF3S1 and eIF4B have been shown to be targets of caspase 3 in the pre-apoptotic phase. Additionally, PABP is degraded during apoptosis [14]. In this case, proteolytic cleavage generally reduces the overall net rate of protein synthesis. In contrast, proteolytic processing of eIF4E has not been described to date. Thus we localized the cleavage site in the eIF4E molecule.

The 23 kDa eIF4E form lacks at most the N-proximal 21 amino acids

It was not possible to enrich large amounts of truncated eIF4E from native samples. Therefore, in an alternative in vitro protease assay, we used a larger amount of the GST–eIF4E fusion protein to obtain Coomassie-Blue-stainable fragments for localization of the cleavage site. Edman degradation was not successful, probably through N-terminal blockage of the truncated eIF4E. We thus performed MS/MS (tandem MS) analysis to identify the cleavage site. A Coomassie-Blue-stained gel is shown in Figure 3(A).

The external substrate (band 1) which is overlaid by endometrial proteins in the P15 and O VX lane and two specific bands (bands 2 and 3), which were only found in protease assays from P15 lysates, are marked. The identity of these bands was determined by Western blotting (Figure 3B). Band 2 represents GST probably linked to the N-terminal fragment of eIF4E and band 3 was the truncated eIF4E. These three bands were recovered and subjected to MS analysis. The results of the MALDI–TOF–TOF MS are summarized in Figure 3(C). In the GST–eIF4E fusion protein, the GST cloning vector and seven fragments of eIF4E were identified (line 1). Fragments 1–4 comprise entirely the N-terminus, up to amino acid 61. Band 2 was identified as the GST cloning vector. Unfortunately, an N-proximal eIF4E fragment was not found in this assay (line 2). However, the protein sequence of the truncated eIF4E (line 3) was established and was found to begin with amino acid 22 (see Supplementary Figure S1). This result suggests that the truncated eIF4E lacks the N-proximal 21 amino acids and the proteolytic cleavage site is located between the residues at position Lys²¹ and Thr²². However, Lys²¹ is targeted by trypsin in the MS analysis. Therefore we cannot exclude the possibility that the eIF4E-specific protease cleaves two to three amino acids further N-terminally. Hence, so far, it is highly speculative to appoint a protease for eIF4E cleavage. For example, it is not clear whether...
EB, P₄ and embryonic signals stimulate the proteolytic processing of eIF4E

Next, we intended to determine whether embryonic signals and/or maternal steroid hormones are involved in the induction of the proteolytic cleavage of eIF4E. However, we failed to establish an endometrial cell culture system in which formation of the truncated eIF4E could be induced. We thus used an animal model for this purpose. The influence of embryonic signals on the expression of the 23 kDa eIF4E was determined in animals where one uterine horn was endoscopically closed before insemination. On P15, the abundance of the 23 kDa eIF4E was high in the endometrium of the embryo-harbouring uterine horn (Figure 4A). The uterine horn without any embryos only had traces of the truncated factor. This observation suggested that embryonic oestrogens stimulate the expression of the eIF4E-processing protease in the P₄-primed endometrium. Therefore we performed steroid-replacement experiments. OVX animals were treated with EB, P₄ or with both hormones (Figure 4B). This approach revealed a single band with a molecular mass of 25 kDa in the endometrium after OVX and predominantly also after EB or P₄ treatment. However, traces of the truncated eIF4E were found in some of the animals treated with EB or P₄, probably reflecting the animal-specific hormonal status. In contrast, the consecutive substitution of both steroid compounds resulted in a strong expression of the truncated eIF4E. Furthermore, only lysates from the animals with the double substitution significantly stimulated the cleavage of the 25 kDa eIF4E when incubated for 90 min. These results are supported by an additional experiment. Only in samples from animals having been substituted with both P₄ and EB could the formation of the truncated eIF4E be reduced by EDTA. This reduction was abolished when Ca²⁺ was added (Figure 4C). All of the other lysates did not significantly stimulate any eIF4E processing even if CaCl₂ was added.

In conclusion, our results indicate that, at the time of implantation when the concentration of P₄ is high [28], the generation of the truncated factor is induced by embryonic signals, probably embryonic 17β-oestradiol.

The truncated eIF4E reduces the repressive function of 4E-BP1 in vitro

We suggest that the truncated eIF4E may play an important physiological role at the time of implantation of the conceptus. Therefore we developed a cell-free translation assay which mimicked the situation in P15 samples to examine the potential regulatory impact of the truncated eIF4E in the translation process.

To this end, an in vitro translation assay with nuclease-treated RRL was performed. The abundance of eIF4G, eIF4A, and of the recombinant eIF4E and 4E-BP1 in the complete and eIF4E-depleted RRLs is shown in Figure 5(A). The complete RRL did not contain endogenous 4E-BP1 or 4E-BP2. Furthermore, the abundance of eIF4G and eIF4A was not strongly influenced reflecting the animal-specific hormonal status. In contrast, the truncated eIF4E or eIF4G were absent or only detected in traces (Figure 5C). Thus the RRL was not heavily contaminated with embryonic 17β-oestradiol (Figure 4A). The uterine horn without any embryos only had several fragments with molecular masses from approx. 100 to 160 kDa. However, eIF4A and PABP were not affected. OVX treatment did not modify any of these factors and OVX-treated RRLs (Figure 5C). Interestingly, eIF4G was cleaved into truncated forms of eIF4E and eIF4G were observed in the P15-treated RRLs (Figure 5C). Thus the RRL was not heavily contaminated with endometrial factors. As expected, after 60 min of treatment only truncated forms of eIF4E and eIF4G were observed in the P15-treated RRLs and in the 4E-BP1-substituted RRL (Figure 5B). Therefore translational stimulation was strictly eIF4E-dependent in our assay.

Next, we modified the RRL to achieve conditions comparable with the situation in the endometrium. Therefore RRL was treated with endometrial lysates from animals after OVX as a control and on P15 to obtain cleaved eIF4E and eIF4G factors. At zero time, truncated eIF4E or eIF4G were absent or only detected in traces (Figure 5C). Thus the RRL was not heavily contaminated with endometrial factors. As expected, after 60 min of treatment only truncated forms of eIF4E and eIF4G were observed in the P15-treated RRLs (Figure 5C). Interestingly, eIF4G was cleaved into several fragments with molecular masses from approx. 100 to 160 kDa. However, eIF4A and PABP were not affected. OVX treatment did not modify any of these factors and OVX-treated RRL stimulated translation comparable with the control with the complete unmodified RRL (Figure 5D compared with Figure 5B). A weaker stimulation was observed in the treated RRL from
Figure 4  Analysis of the influence of embryonic signals and steroid replacements on the expression of the truncated eIF4E

(A) The abundance eIF4E is shown in the embryo-containing (P) and non-embryo-containing (NP) uterine horn on P15 of the same animal (B) OVX animals were treated with EB and P₄ as indicated and the samples were probed for the abundance of internal eIF4E directly (zero time) or after 90 min of incubation. The bar chart shows the ratio of the 25 kDa eIF4E (black) and the 23 kDa form (white). Values are means ± S.E.M. (n = 6). **P < 0.05 between the 25 kDa and 23 kDa forms of eIF4E. (C) Analysis of Ca²⁺-dependent proteolytic cleavage of internal eIF4E after steroid replacement and incubation for 90 min at 37°C with Ca²⁺, EDTA or both compounds.

Figure 5  Analysis of modified RRL and the in vitro translation assay

(A) The RRL was analysed for the abundance of eIF4G, eIF4A, eIF4E, 4E-BP1 and 4E-BP2. Shown are the complete RRL (RL), the m⁷-GTP-Sepharose-treated eIF4E-depleted RRL (−4E), the corresponding supernatant (P), the eIF4E-depleted RRL substituted with recombinant eIF4E (+4E) and the complete RRL substituted with 4E-BP1 (+BP1). (B) The translation products are shown after 0, 15 and 30 min as obtained from complete, eIF4E-depleted, eIF4E-substituted and 4E-BP1-substituted RRL with Xef1 mRNA as a template, as indicated. (C). The abundance and cleavage of eIF4G, eIF4E, eIF4A and PABP of the OVX- and P15-modified RRL were monitored after 0, 30 and 60 min. (D) After 60 min of treatment, the lysates were used for in vitro translation (IVT) of capped polyadenylated Xef1 mRNA. The translation products are shown in complete RRL treated with endometrial lysates after OVX or on P15 and substituted with recombinant 4E-BP1 as indicated. (E) Quantification of the analysis of eIF4E-dependency from (B) and endometrial effects from (D). The absorbance of the bands of the translation products obtained by in vitro translation was measured in RRLs treated, as indicated. rec., recombinant; rel., relative.
fractions from beads saturated with (the beads was also confirmed by Western blotting. The input (in), the bound (B) and unbound lysates after OVX and on P15, and eIF4E binding was monitored. Equal His–4E-BP1 binding to His-tagged 4E-BP1 bound to Ni-NTA magnetic agarose beads was incubated with endometrial precipitated fractions (eIF4E-IP) and the corresponding supernatant. The samples were probed to immunoprecipitation with immobilized eIF4E antibody. The input is compared with the precipitates with the corresponding supernatant in OVX, P15 and 4E-BP1-substituted RRLs (Figure 6A). This

...impaired in this assay.

To analyse the altered eIF4E–4E-BP1 interaction in more detail, we performed a protein interaction assay with recombinant His-tagged 4E-BP1 bound to Ni-NTA beads (Figure 6B). This approach showed that, whereas the 25 kDa form of eIF4E binds to 4E-BP1 (43 ± 2.3 %), the binding of the truncated factor is remarkably reduced (14.6 ± 4.3 %). As a control, the blot was reprobed with an anti-His antibody to ensure equal 4E-BP1 binding to the Ni-NTA resin (Figure 6B, lower panel).

P15. In contrast, OVX treatment with 4E-BP1 substitution did not stimulate translation at all. This inhibitory effect of 4E-BP1 was slightly, but noticeably, reduced in the RRL treated with endometrial lysate from P15. The quantification of these results (Figure 5E) shows the eIF4E-dependency of translation (left-hand panel), the inhibitory effect of 4E-BP1 and the partial relief of 4E-BP1 inhibition in the P15-treated RRL (right-hand panel).

We did not, however, observe a complete repression of translation in P15-treated RRL. Only a reduction (~40 %) of translation efficiency compared with the OVX-treated samples was observed. This is in accordance with results showing that only RRLs containing endogenous competitor mRNA or which were partially depleted of ribosomes show a cap-poly(A)-mediated synergic effect, whereas in other cases this effect was only additive [36].

Truncated eIF4E and eIF4G forms alter the dynamic of eIF4E/4E-BP1 binding and impair eIF4G–PABP interaction

To analyse the formation of the eIF4E complex and PABP binding in the cell-free translation assay, immunoprecipitations with an immobilized anti-eIF4E antibody were performed directly after the in vitro translation reaction. In this experiment, we compared the input and the precipitates with the corresponding supernatant in OVX, P15 and 4E-BP1-substituted RRLs (Figure 6A). This approach confirmed the stable abundance of the truncated eIF4E and eIF4G in the P15-treated RRL. In the OVX-treated control, eIF4F was distinctly formed, as shown by the fact that eIF4G and eIF4A was highly present in the precipitates and only traces remained in the supernatant. Moreover, PABP bound to the complex. As expected, in the 4E-BP1-substituted OVX-treated RRL, the formation of eIF4G and PABP binding was significantly reduced. A different observation was made in the P15-treated RRL. Here, eIF4F was formed, but PABP did not bind to the complex. This indicates that the truncated eIF4E had lost the N-terminally located PABP-binding site. A different behaviour was also observed in the 4E-BP1-substituted P15 assay. No significantly reduced eIF4F formation was observed in comparison with non-substituted P15 RRL. This points to a restricted 4E-BP1 function. Indeed, we observed reduced binding of 4E-BP1 to the truncated eIF4E. Most of the 4E-BP1 molecules remained in the supernatant. Likewise, PABP binding was impaired in this assay.

Regarding the regulatory function of eIF4E in translational initiation, it is known that binding of apo-eIF4E to the eIF4G enhances its affinity to the cap [37–39]. Moreover, cap binding enhances the affinity for eIF4G and 4E-BP1 and 4E-BP2 [38,40,41]. eIF4G and its competitors 4E-BP1 and 4E-BP2 bind to the dorsal surface of eIF4E, near the N-terminal region which is located distal to the cap-binding pocket [42]. The N-terminal segment comprises Gln10 and is a unique flexible domain which is strongly conserved between human, mouse, rat and rabbit [23]. Considering the regulatory impact of the N-proximal segment of eIF4E, interaction analysis with recombinant N-terminally truncated eIF4E (Δ33 residues) revealed that the binding dynamic of eIF4E to 4E-BP1 and 4E-BP2 depends on the interaction state [24]. Kinetic-state SPR (surface plasmon resonance) analysis revealed an approx. 2-fold increase in the apparent association rate constant and a ~20-fold decrease in the dissociation rate constant when the truncated eIF4E was compared with the full-length counterpart. In contrast, in a steady-state fluorescence analysis, the association constant was found to decrease by a factor of four. Hence Tomoo et al. [24] discuss positive and negative contributions of the N-terminal region in 4E-BP1 and 4E-BP2 binding. They describe the N-terminal region of eIF4E as a repressor for the association/dissociation with 4E-BP1 and 4E-BP2. It stabilizes the complex in an equilibrium state and blocks 4E-BP binding in a non-steep kinetic state. However, more recent SPR analysis by this group [25] revealed that the m’GTP–Δ16 eIF4E binary complex has more or less the same binding affinity for 4E-BP1 and 4E-BP2 compared with the full-length eIF4E. In contrast, the complex with Δ26 eIF4E had a reduced association rate for 4E-BP1 and 4E-BP2, whereas with Δ33 eIF4E the dissociation rate was reduced. These results indicate that deletions between 16 and 26 amino acid residues reduce the affinity of the m’GTP–eIF4E binary complex for 4E-BP1, which is in accordance with our present observations.

Little information is available comparing the interaction of the full-length factor with truncated forms of eIF4E and eIF4G. Studies on yeast eIF4E, which has an ~30 % identity with mammalian eIF4E, showed that the N-terminus of eIF4E is required for tight binding to eIF4G and maintaining a long-term
eIF4E complex. Deletion of 20 residues did not influence binding, whereas longer deletions (30–35 residues) significantly reduced eIF4E binding to the eIF4G peptide [26,38].

Regarding the scaffold protein eIF4G, binding sites for components of eIF4F and for PABP have been identified to be located in different domains [43]. In the human eIF4G factor, the PABP- and eIF4E-binding sites are located in the N-terminal segment (amino acids 172–200 in PABP; amino acids 557–646 in eIF4E). The eIF4A-binding sites were found in the central and C-terminal regions (amino acids 712–916 and 1241–1356). We detected the N-terminally truncated forms of eIF4G in the P15-treated RRL. Our results additionally showed that, in contrast with OVX, PABP binding to eIF4G was impaired in RRL treated with P15 endometrial lysates. This indicates a decoupling of the PABP from the eIF4E-binding site. That means the PABP site is not present in the N-terminal truncated eIF4G which can be precipitated with the immobilized eIF4E antibody. The estimated apparent size of the stable C-terminal fragments of eIF4G was 100–160 kDa, but the sequence of the N-terminal segment of rabbit eIF4G is not well established. A database search indicates that the N-terminus of the rabbit factor coincides with position 196 of the human eIF4G factor. However, we suggest that, in RRL, the N-terminal region of eIF4G is quite similar to its human counterpart. For instance, eIF4G in RRL was shown to have an apparent molecular mass of ∼220 kDa [44] and our N-terminal eIF4G antibody maps around Gly188, which is located within the PABP-binding site. Additionally, the eIF4G–PABP interaction has been described in unmodified RRL [36,45].

Truncated eIF4E and eIF4G forms provide an alternative regulation mechanism of translational initiation during implantation

It has been shown that either cap or poly(A) stimulate translational initiation in vivo alone or synergistically [46]. Thus the truncated eIF4E and eIF4G described in our present study allow three different scenarios of regulation of translational initiation in P15 samples. These are: (i) reduced inhibitory impact of 4E-BP1 and 4E-BP2; (ii) impaired eIF4G–PABP interaction; and (iii) altered interaction between the truncated eIF4E and eIF4G.

The different interactions of the factors are summarized in Figure 7. Depicted is a highly schematic representation of eIF4E and eIF4G with the presumed cleavage sites marked by arrows (Figure 7A). Although the investigations were performed with recombinant 4E-BP1, the results might also apply for 4E-BP2 due to its similar binding property [25,41]. Formation of the eIF4F complex and PABP binding is dependent on 4E-BP1 abundance after OVX (Figure 7B), whereas such a pronounced effect of 4E-BP1 was not observed during the interaction of the truncated factors on P15. Moreover, PABP1 binding is impaired at this developmental stage. These different interactions have different impacts on translational initiation. On the one hand a general reduction might be suggested by the impaired ‘closed loop’ formation of the mRNA. On the other hand, initiation could be stimulated by a reduced efficacy of the repressor 4E-BP1 even in situations where this protein is hypophosphorylated.

Given the tight spatial restriction of the occurrence of the truncated eIF4E only at the site of implantation, we propose that the truncated factor contributes to establish the non-invasive placenta which is found in pigs. Such an assumption is supported by the fact that, in particular, the truncated form of eIF4E was not found in ongoing pilot experiments (K. Wollenhaupt and W. Tomek, unpublished work) in species with invasive placenta, such as the mouse, and these endometrial lysates did not cleave exogenous substrates. Furthermore, when porcine embryos are transferred to ectopic sites, the trophoblast becomes invasive [47,48]. This results in erosion of the adjacent epithelium. Therefore the truncation of eIF4E and eIF4G might be crucial for the expression of factors favouring non-invasive over invasive implantation. Thus we envisage that the truncated factors differentially regulate a subset of mRNA moieties, even under conditions of persistent hypophosphorylation of 4E-BP1 and 4E-BP2. Mechanistically, these mRNAs might share peculiar properties in their 5′-UTR and quite possibly their activation might not strictly depend on poly(A) [13,49,50].

AUTHOR CONTRIBUTION

Karim Wollenhaupt and Wolfgang Tomek designed and co-ordinated the study, performed the protein analysis and wrote the manuscript. Klaus-Peter Brüssow performed all of the veterinary treatments. Cell separation and culture were performed by Ute Tiemann and Kati Reinke, and the nucleic acid analysis was conducted by Hans-Martin Seyfert. The MS analysis was performed by Dirk Albrecht. All authors read and proofread the final manuscript.
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SUPPLEMENTARY ONLINE DATA

Natural occurrence and physiological role of a truncated eIF4E in the porcine endometrium during implantation

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EXPERIMENTAL

Materials

Anti-CTBI (actin β chain) was purchased from Sigma. Rabbit polyclonal antibodies against phospho-eIF4E, 4E-BP1, N-terminal elf4G, and PABP1, HRP (horseradish peroxidase)-conjugated anti-rabbit antibody, and monoclonal antibodies against GST and His were purchased from Cell Signaling Technology. The monoclonal antibody against elf4E was obtained from BD Transduction Laboratories, and the secondary HRP-conjugated anti-mouse antibody was from Jackson ImmunoResearch Laboratories. Protein A/G PLUS-agarose, immobilized monoclonal anti-elf4E-agarose and His-tagged 4E-BP1 protein were purchased from Santa Cruz Biotechnology. Ni-NTA magnetic agrose beads were from Qiagen. m7-GTP–BP1 protein were purchased from Santa Cruz Biotechnology. The monoclonal antibody against eIF4E was obtained from BD Transduction Laboratories, and the secondary HRP-conjugated anti-rabbit antibody, and monoclonal antibodies against GST and His were purchased from Cell Signaling Technology. The monoclonal antibody against eIF4E was obtained from BD Transduction Laboratories. The monoclonal antibody against elf4E was obtained from BD Transduction Laboratories. The monoclonal antibody against eIF4E was obtained from BD Transduction Laboratories.

RNA analysis and Northern blotting

TRIzol® was used according to the manufacturer’s instructions (Invitrogen) to extract RNA from the tissues [1]. RNA to be used for Northern blotting was purified further using CsCl gradient centrifugation, then denatured and resolved on 1.2% agarose gels containing 2.2 M formaldehyde essentially as described previously [2]. A portion (10 µg) of total RNA was loaded into each slot. After the separation, the gels were soaked in 20× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate; twice for 15 min) and RNA was transferred on to Hybond N (Amersham Biosciences) using 20× SSC as the transfer buffer. Filters were rinsed twice in 2× SSC and baked (2 h at 80°C). Hybridization probes were generated by labelling 25 ng of the cloned cDNA segments with [γ-32P]dCTP using the Mega Prime DNA labelling kit (Amersham Biosciences) according to the manufacturer’s instructions. Chromatography over Mini Quick Spin columns (Roche) separated unincorporated radiocleotides from the labelled probes. Filters were pre-hybridized for 1 h at 68°C in ExpressHyb solution (BD Biosciences). Probes were denatured (100°C, 5 min), snap-cooled on ice and added to a pre-warmed (68°C) ExpressHyb solution. Hybridization was for 1 h at 68°C. Post-hybridization washes consisted of three rinses at room temperature (2× SSC containing 0.05% SDS for 10 min). Stringent washes (2×15 min) were conducted at 50°C in 0.1× SSC containing 0.1% SDS. Radioactively labelled bands were detected after 5 days of exposure to an intensifier screen with a STORM PhosphorImager (Molecular Dynamics). Generation of cDNA using Superscript (H-; Invitrogen) reverse transcriptase and touch-down PCR-amplification procedures were generally as described previously [3]. Briefly, cDNA was generated from 1.5 µg of total RNA using the Superscript II reverse transcriptase system (Invitrogen), virtually as recommended by the manufacturer. The cDNA was purified with the High Purification kit (Roche) and finally eluted in 50 µl of water. A portion (5 µl) was then used for the first round of PCR amplification. PCR amplifications were performed in 50 µl assays, using the Fast Start Taq DNA Polymerase kit (Roche). Touch-down PCR was used throughout. During the first 11 cycles, we lowered the annealing temperature from 72°C to 60°C in 1°C decrements (1 min denaturation at 95°C, 1 min annealing, 3 min extension at 72°C). Next, we applied 30 cycles with a constant annealing temperature of 60°C. Sequences for oligonucleotide primers were derived from evolutionarily conserved regions of the bovine cDNA sequence of this factor (GenBank® accession number AF257235). We used the oligonucleotide eF4 r1 (5’-GAGAATACCTCAGAAGGTTGTCTTC-3’) to specifically prime the generation of the cDNA from the elf4E factor. This sequence motif is located immediately downstream of the translational stop codon. Oligonucleotide primers eF4 r1 (5’-GAGAATACCTCAGAAGGTTGTCTTC-3’) and eF4 r3 (5’-ATGGGACTGATAACCACTACTAC-3’; reverse) were used to amplify most of the coding region of the porcine elf4E factor. Primer eF4 r2 (5’-GAAACGGAAACCCACTAC-3’) was combined with eF4 r3 for a second nested PCR amplification. This product was cloned into the pGEMTeasy vector and subsequently sequenced.

RESULTS

The 23 kDa elf4E is not a splicing variant of the prototypical elf4E

We analysed whether the 23 kDa elf4E was encoded by an alternatively spliced truncated mRNA. To this end, we first had to establish the cDNA sequence of the porcine factor covering almost the entire coding part of the mRNA sequence (GenBank® accession number AM419457; Figure S1). RNA extracted from liver tissue was used for this purpose. Amplification primers were derived from sequence areas of the elf4E-encoding gene which are conserved in cattle, mouse and humans. Forward and reverse primers were placed on the presumed exon one

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Alterations in the porcine cDNA sequence (GenBank® accession no. AM419457) are indicated. The exchanged bovine nucleotides (GenBank® accession no. AF257235) are noted above the porcine eIF4E cDNA sequence. The porcine cDNA sequence was established from position 31 to position 575 of this sequence and the primers are underlined. The amino acid sequence of the porcine eIF4E is given. The amino acids found exchanged in the porcine factor are labelled in bold (bovine Gly124 replaced by serine; bovine Glu174 by aspartate). The N-terminal flexible region is in bold.

and the terminal exon seven of the porcine gene. The primers comprised the translational start ATG codon (forward) and the reverse primer was placed in the vicinity of the translational stop codon. We retrieved 545 nucleotides of the coding region of the porcine factor. The DNA sequence is highly homologous (>97%) with that encoding the orthologous bovine or human factors. When compared with the bovine sequence, the porcine sequence featured 16 nucleotide exchanges, resulting in two exchanged amino acid residues (Figure S1). Using the cloned cDNA as a probe to detect the eIF4E-encoding mRNA in Northern blots revealed a single predominant band in RNA preparations retrieved from endometrium on P15 and from animals after OVX (Figure S2A). The band runs just below the 18S rRNA moiety, whereas ACTB runs just above the 18S rRNA. ACTB mRNA was used as a control to validate approximately equal loading of total RNA in all of the samples. The results suggest that the eIF4E-encoding mRNA contains ~1800 nucleotides. This size is in agreement with previous observations that the murine eIF4E1 mRNA from various tissues coding for the 25 kDa form of eIF4E has a length of ~1.8 kb [4].

The molecular mass difference between the prototype and truncated eIF4E factors is approx. 2 kDa or 15–20 amino acid

Figure S1 Alterations of the porcine compared with bovine eIF4E cDNA sequence

Alterations in the porcine cDNA sequence (GenBank® accession no. AM419457) are indicated. The exchanged bovine nucleotides (GenBank® accession no. AF257235) are noted above the porcine eIF4E cDNA sequence. The porcine cDNA sequence was established from position 31 to position 575 of this sequence and the primers are underlined. The amino acid sequence of the porcine eIF4E is given. The amino acids found exchanged in the porcine factor are labelled in bold (bovine Gly124 replaced by serine; bovine Glu174 by aspartate). The N-terminal flexible region is in bold.

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The molecular mass difference between the prototype and truncated eIF4E factors is approx. 2 kDa or 15–20 amino acid

Figure S2 Northern blotting and cDNA analysis

(A) Northern blot analysis of eIF4E1 mRNA (left-hand panel) from endometrial samples derived on P15 (two independently generated samples) or after OVX as indicated. As a control the membrane was reprobed for ACTB mRNA (right-hand panel). (B) Analysis of eIF4E1-specific RT–PCR products by agarose gel electrophoresis. RNA was isolated from endometrial samples generated after OVX and on P15. © The Authors Journal compilation © 2010 Biochemical Society
residues. Hence an mRNA molecule encoding the truncated factor might be shortened by 60 nucleotides at most compared with the mRNA moiety encoding the prototype factor. This difference might not be detected unambiguously in a Northern blot analysis of the entire mRNA comprising 1800 nucleotides. Hence we used RT–PCR (reverse transcription–PCR) to analyse only the coding region of the eIF4E mRNA in more detail. We used RNA isolated from endometrial tissue after OVX and on P15 (from the same samples as depicted in Figure 1A of the main text) and amplified most of the coding region of the eIF4E mRNA. RT–PCR generated cDNAs of the same size (599 bp including primers; encoding amino acids 1–199 of the entire 217 amino-acid-comprising protein) from both RNA samples (Figure S2B). This length of the amplicon characterizes the mRNA encoding the prototypical eIF4E variant of the protein. However, this variant of the protein was almost absent on P15. In contrast, the 23 kDa variant of the protein was prominently expressed on P15. We subcloned the amplificates from this analysis and sequenced ten clones derived from the RNA from either tissue. All 20 sequences were identical, conforming to the prototype cDNA sequence. These results together show that there is no indication for the occurrence of an alternatively spliced mRNA encoding the truncated factor.

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