Regulation of protein synthesis by eIF4E phosphorylation in adult cardiocytes: the consequence of secondary structure in the 5'-untranslated region of mRNA

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In adult cardiocytes, eIF4E (eukaryotic initiation factor 4E) activity and protein synthesis are increased concomitantly in response to stimuli that induce hypertrophic growth. We tested the hypothesis that increases in eIF4E activity selectively improve the translational efficiency of mRNAs that have an excessive amount of secondary structure in the 5'-UTR (5'-untranslated region). The activity of eIF4E was modified in primary cultures of adult cardiocytes using adenoviral gene transfer to increase either the amount of eIF4E or the extent of endogenous eIF4E phosphorylation. Subsequently, the effects of eIF4E on translational efficiency were assayed following adenoviral-mediated expression of luciferase reporter mRNAs that were either 'stronger' (less structure in the 5'-UTR) or 'weaker' (more structure in the 5'-UTR) with respect to translational efficiency. The insertion of G + C-rich repeats into the 5'-UTR doubled the predicted amount of secondary structure and was sufficient to reduce translational efficiency of the reporter mRNA by $48 \pm 13\%$. Translational efficiency of the weaker re-

porter mRNA was not significantly improved by overexpression of wild-type eIF4E when compared with the stronger reporter mRNA. In contrast, overexpression of the eIF4E kinase Mnk1 [MAP (mitogen-activated protein) kinase signal-integrating kinase 1] was sufficient to increase the translational efficiency of either reporter mRNA, independent of the amount of secondary structure in their respective 5'-UTRs. The increases in translational efficiency produced by Mnk1 occurred in association with corresponding decreases in mRNA levels. These findings indicate that the positive effect of eIF4E phosphorylation on translational efficiency in adult cardiocytes is coupled with the stability of mRNA.

Key words: cardiocyte, eIF4E (eukaryotic initiation factor 4E), hypertrophy, Mnk1 (MAP kinase signal-integrating kinase 1), protein synthesis, translation.

INTRODUCTION

Adult cardiac muscle cells (cardiocytes) are terminally differentiated and, therefore, growth of the myocardium occurs predominantly via mechanisms that trigger cellular hypertrophy [1]. An extensive amount of evidence has established that protein synthesis is regulated during cardiocyte hypertrophy by adaptive increases in both the activity and the amount of translation machinery, which includes ribosomes, tRNA, initiation and elongation factors [2]. During the initial phase of hypertrophic growth, the rate of protein synthesis is accelerated by increasing the translational efficiency of protein synthesis [3,4]. Efficiency, calculated by dividing the rate of protein synthesis by ribosome content, is defined as the utilization of existing translation machinery in the cell. In all cell types, translational efficiency is controlled at the initiation step, specifically binding of the 43 S pre-initiation complex (40 S ribosome and its associated components) to the 5'-end of mRNA [5]. Whether at steady state or during periods of growth, translational efficiencies of individual mRNAs are determined by their ability to compete effectively for a rate-limiting pool of ribosomes and to form subsequently an initiation complex.

Binding to 43 S pre-initiation complexes is determined by *cis*-acting elements intrinsic to each mRNA and by the activity of *trans*-acting proteins such as eIF4F (eukaryotic initiation factor 4F). Examples of *cis*-acting elements in the 5'-UTR (5' untranslated region) of mRNA are the m⁷Gppp (7-methylguanosine) cap, the presence of upstream AUG codons, the sequence context of the AUG start codon and stable secondary structures produced by G:C base pairing [6,7]. Most capped mRNAs are capable of efficient translation because the start codon is positioned in a favourable context and because the amount of secondary structure in their respective 5'-UTRs is minimal [8]. Conversely, a subset of mRNAs has 5'-UTRs that are longer and/or have a high G + C content, which increases the incidence of secondary structures such as stable hairpin conformations [6]. The relative translational efficiencies of these particular mRNAs are diminished because the processes involved in initiation are usually sensitive to the amount of secondary structure in the 5'-UTR.

eIF4F functions as a *trans*-acting factor that promotes binding of mRNA to the 43 S pre-initiation complex [5]. The eIF4F complex consists of three core proteins: eIF4E, which binds to the m⁷Gppp cap on mRNA; eIF4A, which functions as an ATP-dependent RNA helicase; and eIF4G, which functions in the

Abbreviations used: CMV, cytomegalovirus; eIF, eukaryotic initiation factor; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, haemagglutinin; LUC, luciferase; m⁷Gppp, 7-methylguanosine; MAP, mitogen-activated protein; Mnk, MAP kinase signal-integrating kinase; NCX-1, Na⁺/Ca²⁺-exchanger promoter; RT, reverse transcriptase; 5'-UTR, 5'-untranslated region; PFU, plaque-forming units; WT, wild-type.

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assembly of eIF4F and its ancillary proteins. Once eIF4E binds to the m⁷Gppp cap, the helicase activity associated with eIF4A is hypothesized to facilitate binding of the 43 S pre-initiation complex to mRNA by alleviating secondary structure in the 5'-UTR. The activity of eIF4F is dependent on the assembly of its core proteins and on its affinity for m⁷Gppp caps [5,9]. Assembly of eIF4F is regulated by a family of eIF4E-binding proteins (4E-BP1–3), which compete with eIF4G for a common binding site on eIF4E, thereby controlling the amount of eIF4E available for eIF4F complex formation [10,11]. 4E-BPs serve as effectors of signalling pathways involved in growth and cellular stress through their phosphorylation on an integrated set of sites that reduce their binding affinity for eIF4E [5,12,13]. Affinity of eIF4F for m⁷Gppp caps is regulated by phosphorylation of eIF4E on Ser-209 by Mnk1 [MAP (mitogen-activated protein) kinase signal-integrating kinases 1] or Mnk2, which incorporate into the eIF4F complex by binding to eIF4G [14,15]. Phosphorylation of eIF4E has been positively correlated with cell growth in many cell types, consistent with the fact that Mnk1 and Mnk2 are effectors for the p38 and ERK (extracellular-signal-regulated kinase) branches of the MAP kinase superfamily [15,16].

Despite positive correlations with cell growth, increases in either eIF4F complex formation or eIF4E phosphorylation are not sufficient to accelerate the rate of total protein synthesis [17–19]. Rather, current evidence indicates that eIF4F has more specific functions in regulating cell growth. One hypothesis is that the helicase activity of eIF4F is required to improve selectively translational efficiency of mRNAs that encode for proteins involved in growth regulation such as transcription factors, proto-oncogenes, growth factors and their receptors [8,20]. Many of these mRNAs are relatively weak with respect to translational efficiency as predicted by the extensive amount of secondary structure and/or other structural features of their respective 5'-UTRs [21]. Another possible function of increasing eIF4F activity is to couple translation with changes in mRNA stability [22]. By altering the interactions between proteins of the initiation complex, increases in eIF4F activity could provide a mechanism for controlling accessibility of enzymes that trigger mRNA degradation such as decapping enzymes and deadenylases [23].

In primary cultures of adult cardiocytes, an increase in workload can be generated by electrically stimulated contractile activity [24]. The rate of total protein synthesis is accelerated in contracting cardiocytes by mechanisms that improve translational efficiency, which correlates with increases in both eIF4F complex formation and eIF4E phosphorylation [25,26]. Using adenoviral gene transfer, we found subsequently that the rate of total protein synthesis is not accelerated by overexpression of eIF4E to increase eIF4F complex formation or by overexpression of Mnk1 to increase eIF4E phosphorylation [19]. Given that eIF4E activity and protein synthesis are increased concomitantly in response to stimuli that induce cardiocyte growth [4,25–28], we tested the hypothesis that changes in eIF4E activity selectively regulate the expression of mRNAs on the basis of secondary structure in the 5'-UTR. The approach was to modify the activity of eIF4E by adenoviral gene transfer and to measure differential effects on the expression of reporter mRNAs that were either 'stronger' (less secondary structure in the 5'-UTR) or 'weaker' (more secondary structure in the 5'-UTR) with respect to translational efficiency. These studies show that overexpression of eIF4E did not significantly improve translational efficiency of the weaker reporter mRNA. In contrast, overexpression of Mnk1 was sufficient to increase the translational efficiency of both reporter mRNAs, independent of the amount of secondary structure in their respective 5'-UTRs. The increases in translational efficiency pro-

Figure 1 Illustration of reporter gene region derived from the pAdTrack shuttle plasmid

*Bam*HI inserts were positioned into the 5'-UTR of NCX/B0 to generate either NCX/B1 or NCX/B4. Each construct was confirmed by sequencing and used to make recombinant adenoviruses as described in the text. E1, adenovirus E1 region; LTR, left-hand inverted terminal repeat. The amount of secondary structure in the 5'-UTR of each reporter was predicted by mfold [35].

duced by Mnk1 were associated with corresponding decreases in mRNA levels.

EXPERIMENTAL

Adult feline cardiocytes in primary culture

The method for isolation of cardiocytes from the left ventricle of adult feline myocardium was approved by the Institutional Animal Care and Use Committee and was performed as described previously [24]. The cardiocytes were plated on to 4-well culture trays (Nunc) that were coated with laminin; the dimensions of each well were 2.5 cm × 6.5 cm. The initial plating density was 3×10^5 cardiocytes/well. After 4 h of incubation, the cardiocytes were rinsed and maintained in serum-free media [29]. The cardiocytes were stimulated to contract by delivering 140 V electrical pulses of alternating polarity through the culture medium via carbon electrodes using a custom-built electrical stimulator as described previously [29]. Non-stimulated cardiocytes were quiescent and used as controls.

Construction of recombinant adenoviruses

Replication-defective, recombinant adenoviruses were used to modify eIF4E activity in cardiocytes as described before [19]. Ad.HA-4E/WT (where HA stands for haemagglutinin and WT for wild-type) was used to express WT eIF4E that contained an HA epitope tag on the N-terminus. Ad.HA-4E/209A was used to express an Ser-209 to Ala-209 mutation of HA-eIF4E. Ad.HA-Mnk1 was used to express wild-type Mnk1, a physiological eIF4E kinase. Ad.HA-Mnk1/T2A2 was used to express an inactive form of Mnk1 by mutating Thr-197 and Thr-202 to Ala as shown before [16]. Reporter adenoviruses were generated by homologous recombination using the AdEasy system [30]. As illustrated in Figure 1, the adenoviruses contained two contiguous genes: LUC (luciferase) under the control of the cardiac-specific NCX-1 (Na⁺/Ca²⁺-exchanger promoter) and GFP (green fluorescent protein) under the control of the CMV (cytomegalovirus) promoter/enhancer element. To construct reporter adenoviruses, the pGL2-derived plasmid NCX-1831 was digested with *Bam*HI to generate a 5.3 kb fragment that contained 1.8 kb of NCX-1 gene, the entire first exon (H1), the first 67 nt of intron 1, LUC coding sequence and poly(A) (polyadenylated) signal [31,32]. This fragment was subcloned into the *Bgl*II site of the pAdTrack shuttle plasmid to generate pNCX/B0. The plasmid was linearized by digestion with *Pme*I and mixed in excess with the adenoviral plasmid pAdEasy-1. Homologous recombination was performed

in a transformed BJ5183 strain of *Escherichia coli*. Recombinant adenoviral DNA was purified, digested with *PacI* and used for transfection of the HEK-293 (human embryonic kidney 293) cell line with LIPOFECTAMINE™ reagent (Gibco/BRL). After a plaque-purification step, adenoviruses were propagated and then purified by caesium chloride gradient centrifugation [19]. Adenovirus titres were determined by plaque assay. This reporter adenovirus is referred to as Ad.NCX/B0 (L. Xu and D. R. Menick, unpublished work).

The secondary structure in the 5'-UTR of LUC mRNA was increased according to the strategy of Koromilas et al. [33]. *Bam*HI repeats [CCGGATCCGG] were inserted into pNCX/B0 immediately upstream of the AUG codon. The oligonucleotide 5'-GATCTCCGGATCCGGA-3' was synthesized, annealed and subcloned into the *Bg*/III site of pNCX/B0 to produce the shuttle plasmid pNCX/B1. The presence of a single *Bam*HI insert was confirmed by DNA sequencing. The plasmid was used to generate Ad.NCX/B1 by homologous recombination as described above.

The oligonucleotide 5'-GATCTCCGGATCCGGCCGGATCCGGCCGGATCCGGA-3' was used to generate the plasmid pNCX/B4. After annealing, duplex DNA was ligated into the *Bg*/III site of pNCX/B0. DNA sequencing revealed a recombinant plasmid that contained four *Bam*HI inserts, which was designated pNCX/B4. The plasmid was used to generate the corresponding adenovirus Ad.NCX/B4 by homologous recombination as described above.

Adenovirus infection procedure

For most experiments, a two-step infection procedure was used. After an overnight incubation, cardiocytes were infected first with either Ad.HA-4E/WT, Ad.HA-4E/209A or Ad.HA-Mnk1. Infections proceeded for 4 h at a moi (multiplicity of infection) of 4, based on plaque assays. The media were changed and the cardiocytes were incubated for 16–18 h to allow for transgene expression of more than 90 % of the cardiocytes [19]. As a control, companion dishes were infected at the same moi with Ad.CMV- β Gal, an adenovirus expressing β -galactosidase protein. The cardiocytes were infected a second time with either Ad.NCX/B0, Ad.NCX/B1 or Ad.NCX/B4 reporter adenovirus. The media were changed after 4 h and cardiocytes were maintained as quiescent or were electrically stimulated to contract for 48 h.

Measurements of LUC and GFP activities

Cardiocytes were rinsed three times in ice-cold PBS and lysed in reporter lysis buffer (Promega, Madison, WI, U.S.A.). The lysates were quick-frozen in liquid N₂ and stored at –70 °C. The lysates were thawed, centrifuged at 12000 g for 10 min and LUC activity was measured using the Luciferase Assay System (Promega). Light emission was measured using a luminometer. To measure GFP fluorescence, aliquots of the lysate were diluted in PBS and measured by fluorimetry. Protein concentration was measured by the bicinchoninic acid method (Pierce). The expression of eIF4E and Mnk1 was determined by Western-blot analysis as described before [19]. Antibody against the HA epitope was purchased from Sigma, eIF4E antibody from BD Transduction Laboratories (Lexington, KY, U.S.A.) and phospho-Mnk1 (Thr-197/-202) antibody from Cell Signalling Technology (Beverly, MA, U.S.A.).

Measurements of mRNA levels

Total RNA was extracted from cardiocytes using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). The RNA samples were treated with

RNase-free DNase and purified using RNeasy Mini Kits (Qiagen, Valencia, CA, U.S.A.). RNA concentrations were quantified by the RiboGreen method (Molecular Probes, Eugene, OR, U.S.A.). The relative amounts of LUC mRNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA were measured by single-step RT (reverse transcriptase)–PCR using the QuantiTect SYBR Green RT–PCR kit (Qiagen) and real-time PCR instrumentation (iCycler; Bio-Rad Laboratories, Hercules, CA, U.S.A.). The RT part of the reaction was performed on dilutions of each RNA sample at 50 °C for 45 min using primers that were specific for either LUC mRNA or GAPDH mRNA. For LUC mRNA, the forward primer started at nt 1116, 5'-GGTTGTG-GATCTGGATACCG-3', and the reverse primer started at nt 1249, 5'-ATCCATCCTTGTCATCAAGG-3'. The forward primer for GAPDH mRNA started at nt 354, 5'-AGGTCATCCCAGAGC-TGAAC-3', and the reverse primer started at nt 491, 5'-CCTGCT-TCACCACCTTCTTG-3'. The reactions were heated for one cycle at 95 °C for 15 min followed by 35 cycles of PCR consisting of: (1) denaturation for 15 s at 95 °C, (2) annealing for 30 s at 60 °C and (3) amplification for 30 s at 72 °C. Melt curves were generated after each run to verify melting temperatures (T_m) of the amplicons. Purity of RT–PCR was confirmed by running the reaction products on 2 % (w/v) agarose gels. In each run, standard curves were generated for a primer set by serially diluting RNA from the CMV- β Gal quiescent group and plotting the values for threshold crossing (C_t) as a function of input RNA. The C_t value of each sample was used to extrapolate relative mRNA concentrations from the standard curves.

GFP mRNA levels were measured in companion samples of RNA by slot-blot analysis as described before [4]. Equivalent amounts of total RNA were blotted on to a nylon membrane and hybridized to a ³²P-labelled cDNA probe for GFP. The signals were detected using a PhosphorImager and quantified by digital image analysis.

RESULTS

Generation of reporter constructs in recombinant adenovirus

Secondary structure in the 5'-UTR of mRNA is a major determinant of translational efficiency, which is hypothesized to regulate the expression of select proteins during growth. To test this hypothesis in adult cardiocytes, recombinant adenoviruses were constructed to express LUC reporter mRNAs with differing degrees of secondary structure in the 5'-UTR. LUC mRNA expression was controlled by the cardiac-specific NCX-1 promoter [34], whereas expression of GFP mRNA was driven by the constitutively active CMV promoter (Figure 1). On the basis of previous studies that mapped the start site of transcription in the NCX gene [31], the 5'-UTR of the NCX/B0 transcript is 229 nt in length, including the first exon and 67 nt of the H1 intron. As predicted by mfold [35], secondary structure in the 5'-UTR has a minimum free energy (ΔG) of –57 kcal/mol (1 cal $\approx$ 4.184 J). The predicted amount of secondary structure in the 5'-UTR was increased by subcloning G + C-rich *Bam*HI inserts into a *Bg*/III site located 38 nt upstream of the AUG codon. A single *Bam*HI insert produced NCX/B1, which has slightly lower ΔG of –74 kcal/mol. In NCX/B4, the 5'-UTR contains a region of four contiguous *Bam*HI inserts that markedly increases the secondary structure as predicted by a ΔG of –112 kcal/mol.

Effects of secondary structure on efficiency of translation in the 5'-UTR

To establish that secondary structure in the 5'-UTR is a determinant of translational efficiency, cardiocytes were infected with

Figure 2 Adenoviral-mediated expression of reporter genes

(A) Effects of secondary structure in the 5'-UTR on reporter gene expression in quiescent and contracting cardiocytes. The LUC/GFP ratios were measured 48 h after infection and represent the means $\pm$ S.E.M. ($n = 4$ experiments). * $P < 0.01$ versus NCX/B0 and NCX/B1 in contracting cardiocytes as determined by ANOVA followed by a Student–Newman–Keuls test. (B) Effects of secondary structure in the 5'-UTR on translational efficiency as calculated by dividing LUC activity by the corresponding level of LUC mRNA. Values are the means $\pm$ S.E.M. for three experiments. (C) Translational efficiency of GFP was calculated by dividing GFP fluorescence by the corresponding levels of GFP mRNA. Values are the means $\pm$ S.E.M. for three experiments.

either NCX/B0, NCX/B1 or NCX/B4 reporter adenovirus. At 48 h post-infection, LUC activity in cardiocyte homogenates was normalized to GFP fluorescence. Figure 2(A) shows that LUC/GFP ratios derived from NCX/B0 and NCX/B1 were the same in quiescent cardiocytes, but LUC/GFP derived from NCX/B4 was lower. These results suggested that the addition of secondary structure to the 5'-UTR of NCX/B4 produced a corresponding reduction in its translational efficiency. When compared with quiescent cardiocytes, electrically stimulated contraction for 48 h increased LUC/GFP ratios derived from NCX/B0, NCX/B1 and NCX/B4 by 2.7 ± 1.4 -, 2.9 ± 0.7 - and 2.2 ± 0.4 -fold respectively (means $\pm$ S.D., $n = 4$). Consequently, LUC/GFP ratios derived from the NCX/B4 adenovirus remained significantly lower in contracting cardiocytes when compared with either NCX/B0 or NCX/B1.

Figure 3 Comparative effects of electrically stimulated contraction on the expression of LUC protein versus LUC mRNA

Values for LUC activity and LUC mRNA in contracting cardiocytes were calculated relative to the corresponding quiescent controls. Values are the means $\pm$ S.E.M. ($n = 3$), except for NCX/B4 mRNA for which the mean and range of two experiments are given.

To measure directly whether translational efficiency of NCX/B4 was lower, a similar set of experiments was performed in which LUC activity per μ g of protein was divided by the corresponding level of LUC reporter mRNA (Figure 2B). LUC mRNA levels were quantified by real-time RT–PCR assays, which were normalized using GAPDH mRNA as an internal control. When compared with NCX/B0, translational efficiency of NCX/B4 was reduced by $48 \pm 13\%$ in contracting cardiocytes (means $\pm$ S.D., $n = 3$). These results confirmed that the decrease in LUC/GFP ratios, derived from NCX/B4, was due to a corresponding reduction in translational efficiency. Figure 2(C) shows that there were no differences in the translational efficiency of GFP as calculated by dividing GFP fluorescence by GFP mRNA. This result is consistent with the fact that each reporter adenovirus contained the constitutively active CMV promoter, which resulted in high levels of GFP mRNA expression.

Figure 3 shows that contraction generated comparable increases in LUC mRNA and LUC protein from all three reporters as determined by normalization to the corresponding quiescent controls. Thus the increases in LUC/GFP in contracting cardiocytes reflected changes in LUC mRNA levels, rather than improved translational efficiency. The increases in LUC mRNA probably occurred by activation of the NCX promoter (L. Xu, C. Kappler, P. Withers and D. R. Menick, unpublished work). This increase in LUC mRNA levels in contracting cardiocytes further suggests that transcriptional regulation of the LUC gene was not compromised by the presence of the stronger CMV promoter element driving GFP expression. Taken together, the results in Figures 2 and 3 demonstrate that translational efficiency was determined by the degree of secondary structure in the 5'-UTR and that intrinsic differences in translational efficiency were maintained as LUC mRNA levels increased in contracting cardiocytes.

Modifications of eIF4E activity in cardiocytes by adenoviral gene transfer

Given the intrinsic differences in translational efficiencies of NCX/B0 and NCX/B4, these reporters were utilized to test whether changes in eIF4E activity differentially regulated LUC expression on the basis of secondary structure in the 5'-UTR. The activity of eIF4E is determined by the amount available for eIF4F

Figure 4 Modifications of eIF4E activity in cardiocytes by adenoviral gene transfer

Cardiocytes were infected with the indicated adenovirus and protein expression was measured after 48 h. **(A)** Expression of HA-tagged eIF4E and Mnk1. The top panel shows a representative Western blot using eIF4E antibody. The positions of endogenous eIF4E and HA-eIF4E are indicated. The middle panel shows exogenous HA-eIF4E and HA-Mnk1 levels as determined by Western-blot analysis using an antibody against the HA epitope. The bottom panel shows the activation state of HA-Mnk1 as determined by Western-blot analysis using phospho-Mnk1 (Thr-197/-202) antibody. The antibody cross-reacted with Mnk2 as indicated. The same results were obtained in three experiments. **(B)** Non-phosphorylated and phosphorylated isoforms of eIF4E resolved by one-dimensional isoelectric focusing and detected by Western blotting using eIF4E antibody. A representative blot is shown. Q, quiescent cardiocytes; S, electrically stimulated cardiocytes; *, reaction of antibody with a non-specific protein. Summary data as quantified by digital image analysis are given below each lane. Values are the means $\pm$ S.E.M. for three experiments.

complex formation and by its affinity for m⁷Gppp caps on mRNA. Using adenoviral gene transfer [19], we demonstrated, in previous studies, that the activity of eIF4E was modified in cardiocytes as follows: (1) HA-4E/WT increased eIF4F complex formation, (2) HA-4E/209A increased eIF4F complex formation in the absence of eIF4E phosphorylation on Ser-209 and (3) HA-Mnk1 increased endogenous eIF4E phosphorylation without increasing eIF4F complex formation. The same infection procedure was utilized in the present studies. Following an 18 h incubation period, cardiocytes were infected a second time with either NCX/B0 or NCX/B4 reporter adenovirus. The cardiocytes were maintained for another 48 h as quiescent or were electrically stimulated to contract at 1 Hz. The Western blots in Figure 4(A) show that HA-4E/WT and HA-4E/209A levels were markedly increased relative to endogenous eIF4E. An antibody against the HA epitope confirmed that the levels of expression were equivalent in quiescent and contracting cardiocytes. A phospho-Mnk1 (Thr-197/-202) antibody demonstrated that HA-Mnk1 was activated in both quiescent and contracting cardiocytes. The slower migrating band was probably Mnk2, which has been shown to cross-react

Figure 5 Comparative effects of modifying eIF4E activity on LUC expression derived from the NCX/B0 reporter

Adenoviral gene transfer was used to generate the four treatment groups as indicated, followed by a second round of infection with NCX/B0 adenovirus. **(A)** Relative LUC activity calculated by normalizing LUC activity per μ g of protein to the β Gal quiescent group. Values are the means $\pm$ S.E.M. ($n = 7$ experiments). * $P < 0.05$ versus the β Gal contracting group as determined by ANOVA followed by a Student–Newman–Keuls test. **(B)** Relative LUC mRNA levels quantified by real-time RT–PCR. Relative LUC mRNA levels were extrapolated from a standard curve of the β Gal quiescent group. Values are the means $\pm$ S.E.M. ($n = 4$ experiments). * $P < 0.05$ versus the β Gal contracting group as determined by ANOVA followed by a Student–Newman–Keuls test. **(C)** Translational efficiencies plotted by dividing the mean values for relative LUC activity shown in **(A)** by the relative levels of LUC mRNA shown in **(B)**.

with the antibody. Figure 4(B) shows that overexpression of HA-Mnk1 increased eIF4E phosphorylation and that the highest percentage of eIF4E phosphorylation occurred in contracting cardiocytes.

Comparative effects of modifying eIF4E activity on reporter gene expression

Effects of modifying eIF4E activity on LUC expression were examined in quiescent and contracting cardiocytes by measuring changes in LUC activity and the corresponding levels of LUC mRNA (Figure 5). In each individual experiment, relative values

for LUC activity or LUC mRNA were calculated by normalizing to the β Gal quiescent group. Figure 5(A) shows that LUC activities derived from NCX/B0 were not significantly different between the four groups of quiescent cardiocytes, which indicated that synthesis of LUC protein was equivalent. The experiments in Figure 5(B) show that the differences in LUC mRNA levels were small in quiescent cardiocytes except for the HA-Mnk1 group, which was $69 \pm 17\%$ lower than β Gal (means $\pm$ S.D., $n=4$). Despite this reduction in LUC mRNA, the effects of HA-Mnk1 on LUC expression were minimal given that LUC activity in quiescent cardiocytes was not significantly reduced relative to the β Gal control.

In contracting cardiocytes, there was an increase in LUC activity in the β Gal group that was not affected by overexpression of either HA-4E/WT or HA-4E/209A (Figure 5A). Direct comparisons with Figure 5(B) demonstrated that the relative levels of LUC mRNA increased concomitantly. Although LUC activity also increased in contracting cardiocytes overexpressing HA-Mnk1, the relative value was significantly lower than that produced by the β Gal group. Figure 5(B) indicated that HA-Mnk1 blunted the contraction-induced increase in LUC activity by reducing the relative level of LUC mRNA.

Translational efficiency is defined as the amount of protein synthesized from a corresponding pool of mRNA. The effects of modifying eIF4E on translational efficiency of NCX/B0 were calculated by dividing relative LUC activities (Figure 5A) by the relative levels of LUC mRNA (Figure 5B). As shown in Figure 5(C), translational efficiency of NCX/B0 was not affected by overexpression of either HA-4E/WT or HA-4E/209A. Furthermore, translational efficiencies were similar in quiescent and contracting cardiocytes, which underscores that contraction regulated LUC activity in these groups by increasing LUC mRNA levels. Although relative levels of LUC mRNA were reduced by overexpression of HA-Mnk1, Figure 5(C) shows that translational efficiency of NCX/B0 was actually increased by a factor of 2.6 in quiescent cardiocytes and by a factor of 2.2 in contracting cardiocytes. Thus relatively more LUC protein was produced from a smaller pool of LUC mRNA.

NCX/B4 was used to determine whether modifications to eIF4E altered LUC expression from a reporter that is weaker than NCX/B0 with respect to translational efficiency (Figure 6). Relative LUC activities in quiescent cardiocytes were not significantly different in any of the four groups. Since LUC mRNA levels were $58 \pm 33\%$ lower in quiescent cardiocytes overexpressing HA-4E/WT and $62 \pm 27\%$ lower in quiescent cardiocytes overexpressing HA-Mnk1 (means $\pm$ S.D., $n=3$), the calculated values for translational efficiency were higher than the β Gal control group (Figure 6C).

In contracting cardiocytes, relative LUC activity derived from NCX/B4 was increased in all four groups, although HA-Mnk1 caused a modest reduction in this response (Figure 6A). Direct comparison with Figure 6(B) indicates that increases in LUC activity in contracting cardiocytes overexpressing β Gal, HA-4E/WT or HA-4E/209A were associated with changes in LUC mRNA levels, rather than improved efficiency of NCX/B4. In contrast, relative LUC activity increased in contracting cardiocytes overexpressing HA-Mnk1 without a corresponding increase in LUC mRNA. As a result, translational efficiency of NCX/B4 was increased by a factor of 3.7 relative to β Gal (Figure 6C).

To monitor infection efficiency, both adenoviruses contained the GFP gene under control of the CMV promoter. GFP mRNA provided a control for measuring the expression of an mRNA that contains a short 5'-UTR consisting of 31 nt. In Figure 7, the effects of modifying eIF4E activity on GFP expression were determined by measuring GFP fluorescence and the corresponding levels of

Figure 6 Comparative effects of modifying eIF4E activity on LUC expression derived from the NCX/B4 reporter

(A) Relative LUC activity calculated by normalizing LUC activity per μ g of protein to the β Gal quiescent group. Values are the means $\pm$ S.E.M. ($n=6$ experiments). (B) Relative LUC mRNA levels quantified by real-time RT-PCR. Values are the means $\pm$ S.E.M. ($n=3$ experiments). * $P < 0.05$ versus the β Gal contracting group as determined by ANOVA followed by a Student–Newman–Keuls test. (C) Translational efficiencies plotted by dividing the mean values for LUC activity shown in (A) by the relative LUC mRNA levels shown in (B).

GFP mRNA. The values for GFP fluorescence, derived from either adenovirus, were relatively constant, consistent with the fact that GFP mRNA was synthesized constitutively. Contraction produced small, but consistent, increases in GFP fluorescence in all four groups, but these did not reach statistical significance. Figure 7(B) shows that relative levels of GFP mRNA were unchanged in quiescent and contracting cardiocytes. Taken together, these results demonstrate that none of the modifications to eIF4E activity affected the translational efficiency of GFP mRNA.

Overexpression of HA-Mnk1 did not produce complete phosphorylation of the endogenous eIF4E pool (Figure 4B), which suggests that Mnk1 could alter translational efficiency independent of its effect on eIF4E phosphorylation. To address whether

Figure 7 Comparative effects of modifying eIF4E activity on GFP expression

(A) Relative GFP expression derived from either NCX/B0 or NCX/B4 reporter adenovirus. GFP fluorescence per μg of protein for each reporter adenovirus was normalized to the βGal quiescent group. Values are the means $\pm$ S.E.M. ($n = 6$ experiments). (B) Relative GFP mRNA levels as quantified by slot-blotting using equivalent amounts of total RNA. The values are the means $\pm$ S.E.M. ($n = 3$ experiments).

the effects of Mnk1 overexpression were due to eIF4E phosphorylation, cardiocytes were infected with an adenovirus expressing a kinase-deficient mutant HA-Mnk1/T2A2. Figure 8(A) shows that overexpression of Mnk1/T2A2 blocked eIF4E phosphorylation in quiescent and contracting cardiocytes, although a small amount of the phosphorylated isoform remained. Figure 8 further shows that relative levels of LUC protein and LUC mRNA were lower in cardiocytes overexpressing Mnk1/T2A2 and that essentially the same results were obtained using either the NCX/B0 or NCX/B4 reporter. These findings indicate that Mnk1 reduced steady-state levels of LUC mRNA by a mechanism that does not involve phosphorylation of eIF4E on Ser-209. However, when compared with wild-type Mnk1, relative levels of LUC protein and LUC mRNA were more than 2-fold higher in the Mnk1/T2A2 group. Overexpression of Mnk1/T2A2 did not prevent the increase in LUC mRNA levels generated by electrically stimulated contraction. As a result, relative translational efficiencies in the Mnk1/T2A2 group of contracting cardiocytes were 1.2 and 1.4 for NCX/B0 and NCX/B4 respectively. These values are similar to βGal controls.

Figure 8 Comparative effects of Mnk1 overexpression

(A) Cardiocytes were infected with the indicated adenovirus and eIF4E phosphorylation was measured after 48 h. Western blot showing non-phosphorylated and phosphorylated isoforms of eIF4E. (B) Relative LUC activity calculated by normalizing LUC activity per μg of protein to the βGal quiescent group. Values are the means $\pm$ S.E.M. ($n = 3$ experiments). (C) Relative LUC mRNA levels quantified by real-time RT-PCR. The values were normalized to relative levels of GAPDH mRNA as measured in companion samples. Values are the means $\pm$ S.E.M.

DISCUSSION

The eIF4F complex is an essential component of the translation machinery that controls the initiation step in both dividing and non-dividing cells. There is substantial evidence that increasing eIF4E activity is required for proliferative growth to regulate selectively the expression of genes involved in the cell cycle [36]. On the other hand, the function of eIF4F in regulating overall cell

size has not been defined, although studies in *Drosophila* indicate that normal growth and development were dependent on phosphorylation of eIF4E on the site equivalent to Ser-209 in humans [37]. It is apparent that changes in eIF4E activity do not function as a general mechanism for triggering cell growth since neither increasing eIF4E availability to promote eIF4F complex formation nor increasing eIF4E phosphorylation to alter cap binding affinity was sufficient to increase total protein synthesis [17,19,38–40]. Given that adult cardiocytes are terminally differentiated and grow by hypertrophy, these cells were ideally suited to test whether changes in eIF4E activity perform a function analogous to that posited for proliferative growth, i.e. controlling the expression of selected mRNAs on the basis of secondary structure in the 5'-UTR.

The 5'-UTRs of human mRNA have an average length of 210 nt and a G + C content of approx. 60 % [20]. The 5'-UTR of NCX/B0 mRNA has a comparable length of 229 nt and a G + C content of 49 %. Considering that its secondary structure is moderate as predicted by a ΔG value of -57 kcal/mol, the features of NCX/B0 generally fit the profile of most of the mRNAs in the cardiocyte. One significant difference is that the 5'-UTR of NCX/B0 contains several upstream AUG codons proximal to the location of the G + C insert. These codons are not positioned in-frame with the LUC coding region nor do they have in-frame stop codons that could produce upstream open reading frames. Thus it is probable that initiation proceeded by a 'leaky scanning' mechanism since none of these AUG codons is flanked by the conserved sequence context of A⁻³ and G⁺⁴ required for proper recognition of the initiation codon during ribosome scanning [7]. The NCX/B4 reporter is identical except for the G + C inserts that double the predicted amount of secondary structure in the 5'-UTR.

Translational efficiency of NCX/B0 was not significantly improved by overexpression of WT eIF4E in the adult cardiocyte. A similar finding was observed using GFP, a constitutively expressed mRNA with a short 5'-UTR that contains a minimal amount of secondary structure. Given that overexpression of eIF4E in adult cardiocytes promotes eIF4F complex formation [19], one possibility is that translational efficiency of these mRNAs is skewed towards their maximal values in the controls. Consequently, the ability to improve translational efficiency further by increasing eIF4E levels would be limited. An alternative possibility is that a need for additional eIF4E was circumvented because initiation occurred mainly by recycling (re-initiation) of translation machinery on mRNAs engaged in polysomes. Although mRNAs require eIF4F for ribosome binding during the process of *de novo* initiation, this requirement may be lost during ensuing rounds of initiation on mRNAs that are active in translation [41]. In either case, these results underscore that increasing the eIF4E levels is not sufficient for accelerating the rate of total protein synthesis. As in other cell types, mechanisms that either increase or sequester eIF4E in cardiocytes have minimal effects on total protein synthesis, despite the fact that corresponding changes in eIF4F complex formation occur in response to growth stimuli, nutritional status or stress [19,38,42,43].

Analysis of 5'-UTR databases has revealed that the best predictive feature for discriminating weakly translated mRNAs is the amount of secondary structure as estimated by free energy of activation which is usually less than -60 kcal/mol [21]. By doubling the amount of secondary structure in the 5'-UTR to -120 kcal/mol, NCX/B4 was transformed into a demonstrably weaker mRNA than NCX/B0 with respect to translational efficiency in both quiescent and contracting cardiocytes. Overexpression of eIF4E did not selectively improve translational efficiency of NCX/B4, suggesting that increases in eIF4E do not directly promote the expression of 'weaker' mRNAs. Given that NCX/B4

is a chimaeric mRNA transcript, it is probable that other structural features of the 5'-UTR render certain mRNAs dependent on increased eIF4E activity for efficient translation. For example, the predicted G:C base pairings of the 5'-UTR are positioned near the AUG start codon; as such the added secondary structure may not have hindered accessibility to the 5'-cap. In the 5'-UTRs of naturally occurring mRNAs, secondary structure is more likely to interfere with ribosome binding when it is situated in the immediate vicinity of the 5'-end [6].

The eIF4E/209A mutation had little effect on gene expression derived from either reporter, a finding which indicates that there is no apparent link between secondary structure in the 5'-UTR and dependence on eIF4E phosphorylation for efficient translation. Since the Ser-209/Ala mutation does not impair incorporation of eIF4E into eIF4F complexes [19], we conclude that the affinity state of the non-phosphorylated form of eIF4E was sufficient to catalyse the initiation step. Other studies support this conclusion: eIF4E containing the Ser-209/Ala mutation restored efficiency of an *in vitro* translation system depleted of eIF4E, whereas overexpression in adult cardiocytes did not affect the rate of total protein synthesis [18,19]. Similar to wild-type, cap binding of eIF4E containing the Ser-209/Ala mutation is probably stabilized as a result of conformational changes produced by its incorporation into eIF4F complexes [44].

Translational efficiencies of both NCX/B0 and NCX/B4 were improved following overexpression of Mnk1 in conjunction with increased eIF4E phosphorylation. These changes in efficiency were associated with corresponding reductions in the size of the LUC mRNA pool, consistent with the concept that translation and mRNA degradation are tightly coupled [22]. One possibility is that eIF4E phosphorylation functions by promoting the release of eIF4F and other components of the initiation complex by reducing affinity for the 5'-cap [45,46]. Following the release of the reporter mRNA from the initiation complex, we hypothesize that the susceptibility of mRNA to degradation was enhanced. Consequently, only reporter mRNAs active in translation would be stabilized, whereas the residual pool of mRNA would be degraded. Studies in yeast have shown that mutations that disrupt cap binding trigger mRNA degradation by an integrated, two-step process which involves removal of the poly(A) tail by the deadenylase PARN and cleavage of the m⁷Gppp cap by decapping enzymes such as Dcp1 [22,47]. The lower affinity state of phosphorylated eIF4E could produce a net effect of destabilizing mRNA by allowing decapping enzymes and deadenylases to interact with other components of the initiation complex such as eIF4G [22]. In contrast with LUC mRNA, the relative levels of GFP mRNA were not significantly reduced following overexpression of Mnk1, although small reductions in GFP mRNA did occur using either of the reporter adenoviruses. The levels of GFP mRNA were sustained by constitutive activity of the CMV promoter driving transcription, which probably compensated for any loss of GFP mRNA caused by degradation.

The finding that overexpression of Mnk1/T2A2 lowered steady-state levels of LUC reporter mRNA indicates that Mnk1 can regulate gene expression by mechanisms that do not involve phosphorylation of eIF4E on Ser-209. Insight into possible mechanisms can be deduced from the fact that Mnk1 interacts with several classes of proteins that function in regulating gene expression [48]. For example, Mnk1 contains a nuclear localization sequence that binds to the nuclear import protein α -importin [49]. Mnk1 is normally cytoplasmic, but overexpression could have facilitated its movement into the nucleus or affected the shuttling of Mnk1 and its associated proteins between the nucleus and cytoplasm. Mnk1 binds to other components of the translation machinery such as eIF4G and p97/NAT-1 [48]. Although these proteins may

not be phosphorylated directly by Mnk1, increasing their interactions with Mnk1 following overexpression could modify their activities, thereby altering translation and/or mRNA stability.

Knauf et al. [17] used a bicistronic reporter construct to show that overexpression of constitutively activated mutations of either Mnk1 or Mnk2 reduced cap-dependent translation by approx. 25 % relative to translation directed by an IRES (internal ribosome entry site). The region between the 5'-cap and the AUG start codon was short and had a minimal amount of secondary structure, typical of an mRNA that is 'strong' with respect to translational efficiency. Transcript levels of the bicistronic reporter were not directly measured, therefore any contribution resulting from the effects of changes in mRNA stability on expression was not addressed. Similar to our findings obtained using GFP, the modest reduction in cap-dependent translation produced by overexpression of Mnk1 was obtained using a reporter mRNA whose synthesis was controlled by the CMV promoter. Constitutive synthesis of the reporter mRNA could sustain the size of the mRNA pool, but could also mask changes in translational efficiency which are coupled with stability. This study demonstrated further that the relative efficiency of cap-independent translation was enhanced following Mnk activation. This finding suggests that IRES-driven mRNAs could be less susceptible to the putative effects of eIF4E phosphorylation.

Overexpression of Mnk1 causes a modest decrease in the rate of total protein synthesis, which may occur if a sustained increase in eIF4E phosphorylation produces a general decrease in cap-binding affinity [17,19]. However, endogenous levels of Mnk are sufficient to increase eIF4E phosphorylation in response to growth stimuli, and a positive correlation between eIF4E phosphorylation and total protein synthesis has been established in adult cardiocytes [26,28]. The reason for this discrepancy is not known, but the extent and duration of eIF4E phosphorylation produced in cardiocytes overexpressing Mnk1 could have exceeded the changes normally required for cell growth. Some degree of Mnk activation is probably involved in cardiocyte hypertrophy, given that Mnk1 is a substrate for ERK. In transgenic mice, overexpression of a constitutively active MEK1 (MAP kinase/ERK kinase) produced sustained activation of ERK1/ERK2 and stimulated cardiac hypertrophy [50]. In adult cardiocytes treated with hypertrophic agonists, ERK signalling pathways are involved in the phosphorylation of 4E-BPs and subsequent formation of eIF4F complexes [28]. A lower and/or transient increase in eIF4E phosphorylation might be able to disrupt initiation and modify the composition of mRNAs active in translation.

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