Protein Kinase C Regulates Internal Initiation of Translation of the GATA-4 mRNA following Vasopressin-induced Hypertrophy of Cardiac Myocytes*

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GATA-4 is a key member of the GATA family of transcription factors involved in cardiac development and growth as well as in cardiac hypertrophy and heart failure. Our previous studies suggest that GATA-4 protein synthesis may be translationally regulated. We report here that the 518-nt long 5′-untranslated region (5′-UTR) of the GATA-4 mRNA, which is predicted to form stable secondary structures (~65 kcal/mol) such as to be inhibitory to cap-dependent initiation, confers efficient translation to monocistronic reporter mRNAs in cell-free extracts. Moreover, uncapped GATA-4 5′-UTR containing monocistronic reporter mRNAs continue to be well translated while capped reporters are insensitive to the inhibition of initiation by cap-analog, suggesting a cap-independent mechanism of initiation. Utilizing a dicistronic luciferase mRNA reporter containing the GATA-4 5′-UTR within the intercistronic region, we demonstrate that this leader sequence confers functional internal ribosome entry site (IRES) activity. The activity of the GATA-4 IRES is unaffected in trans-differentiating P19C16 cells, however, is strongly stimulated immediately following arginine-vasopressin exposure of H9c2 ventricular myocytes. IRES activity is then maintained at submaximal levels during hypertrophic growth of these cells. Supraphysiological Ca2+ levels diminished stimulation of IRES activity immediately following exposure to vasopressin and inhibition of protein kinase C activity utilizing a pseudosubstrate peptide sequence blocked IRES activity during hypertrophy. Thus, our data suggest a mechanism for GATA-4 protein synthesis under conditions of reduced global cap-dependent translation, which is maintained at a submaximal level during hypertrophic growth and point to the regulation of GATA-4 IRES activity by sarco(ER)-reticular Ca2+ stores and PKC.

The family of GATA transcription factors regulates differentiation, growth, and survival of a wide range of cell types (1). GATA-4 is a zinc-finger-containing transcription factor, which is expressed, in various mesoderm-derived tissues such as the lung, liver, gonad, and gut where it regulates tissue-specific gene expression (2, 3). Consistent with the observed expression patterns, targeted disruption of GATA-4 in mice has elucidated important functions in each of these tissues. GATA-4 is also essential for proper cardiac morphogenesis and also plays an important role as one of the transcriptional factors, which regulates the hypertrophic response in cardiac myocytes (4–6).

Several studies suggest that GATA-4 can be regulated at both the transcriptional level and by phosphomodulation during hypertrophy (1, 7, 8). In cultured neonatal cardiomyocytes, electrical pacing-induced hypertrophy is associated with a significant increase in GATA-4 mRNA, suggesting a mechanism whereby total GATA-4 content is up-regulated during hypertrophy (9). Increases in GATA-4 phosphorylation mediated by Raf/Mek/Erk signaling subsequent to hypertrophic agonist administration have also been demonstrated (10). Our previous data (11) suggested that the GATA-4 mRNA is also regulated at the translational level and we undertook this study to evaluate the possibility that such control may play a role in augmenting GATA-4 potency during differentiation or hypertrophy in cardiac myocytes.

Most mRNAs contain 5′-UTRs that are relatively unstructured and typically less than 100 nucleotides in length, which allows efficient cap-dependent translational initiation (12). However, the leaders of some cellular mRNAs are long, highly structured, and can contain multiple upstream AUG or CUG codons such that scanning ribosomes are unlikely to efficiently initiate translation. In a number of these mRNAs, translation initiation is mediated by cap-independent mechanisms via an internal ribosome entry site (13). IRES-mediated translation initiation can occur during a variety of physiological conditions and has been reported to promote initiation.
for several mRNAs during cell-cycle progression, differentiation, apoptosis, and during stress responses (14–18).

Previously, we identified several mRNAs which either increased or continued to be well translated during conditions when cap-dependent translation initiation was inhibited (11, 19). One of these mRNAs, GATA-4, increased its translational efficiency markedly following exposure of cells to the mTOR inhibitor rapamycin. Treatment with rapamycin results in the global inhibition of cap-dependent translation via a blockade to the formation of productive eIF4F initiation complexes (20).

The human GATA-4 transcript has a 518 nucleotide 5'-UTR and contains 18 upstream initiation codons (21). This prompted us to investigate the possibility of whether this mRNA could initiate translation in a cap-independent fashion and contain an IRES within its leader.

Because GATA-4 has a prominent role in the regulation of muscle cell-specific differentiation and IRES-mediated translation may be enhanced during differentiation (22, 23), we analyzed the ability of this leader to mediate internal initiation during this process. We utilized the P19CL6 cell line model to investigate the possibility that the GATA-4 mRNA could initiate IRES-mediated translation during differentiation. The P19CL6 cell line is a clonal derivative of the P19 embryonal carcinoma cell line which efficiently differentiates into beating cardiomyocytes and produces characteristic muscle proteins (24). GATA-4 is expressed in these cells, is induced upon differentiation, and is required to trans-activate muscle-specific genes (25). Additionally, because GATA-4 is known to be required for cardiac hypertrophy we determined whether IRES-mediated translation initiation of GATA-4 mRNA could occur following vaso-pressin-induced hypertrophy of H9c2 cardiomyocytes.

We report here that the GATA-4 5'-UTR contains IRES activity, which is stimulated during hypertrophy but not during differentiation. Our data also suggest that GATA-4 IRES activity is enhanced coincident with elevated eIF-2α phosphorylation during hypertrophy. We propose that IRES-mediated translational initiation of GATA-4 mRNA allows continued protein synthesis under conditions of reduced cap-dependent initiation potential during the initial response to vasoressin and continues during hypertrophic growth. Moreover, our data suggest that sarco (endo)plasmic Ca2+ flux may play a role in the stimulation of GATA-4 IRES activity through a protein kinase C-dependent pathway during hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and DNA Constructs**—All cell lines were obtained from ATCC (American Type Culture Collection) and maintained in medium supplemented with 10% fetal bovine serum. P19CL6 cell lines expressing the dicistronic reporter mRNAs were generated by co-transfection with pCDNA3.1 and pRGATA-4F followed by G418 selection and subsequent screening for stable expression by Northern analysis. Stable clones were induced to differentiate under adherent conditions by plating at a density of 3.7 × 10^5 cells in a 60-mm culture dish with 1% dimethyl sulfoxide. The medium was changed every 2 days. Days of differentiation were numbered consecutively, with the first day of Me2SO treatment as day 0. The protein kinase C cell permeable myristoylated pseudosubstrate peptide 20–28 (12 μM) and nifedipine (1 μM) was obtained from Calbiochem, and Arg-vasopressin (1 μM) was from Sigma.

5'-RACE analysis of the GATA-4 mRNA was performed on total RNA from H9c2 or HeLa cells using the 5'/3'-RACE kit, 2nd Generation, as described by the manufacturer (Roche Applied Science). To construct the dicistronic reporters, the entire human GATA-4 5'-UTR (accession number NM_002052) was amplified from IMAGE clone 57744870 and inserted into the NcoI site of pRF (kindly provided by A. Willis, University of Nottingham, UK). The construction of pRmycF has been described previously (19). The GATA-4 5'-UTR was also inserted into a promoterless version of pRF (26), (–)pRF (kindly provided by J.-T. Zhang, Indiana University) to generate –(p)RGATA-4F. A 46-bp HindIII-BamHI fragment containing a hairpin structure containing an 18-bp stem (ΔG = −61 kcal/mol) was liberated from pSP64-hp7 (27) (kindly provided by L. Maquat, Roswell Park Cancer Institute) and inserted immediately upstream of the Renilla ORF in pRGATA-4F to generate pRGATA-4F. To generate the constructs used in the monocistronic analysis of RNAs in vitro, the firefly luciferase ORF or the GATA-4 5'-UTR–firefly luciferase ORF sequences were amplified from pRGATA-4F and subcloned into the pSP64 poly(A) vector (Promega) to generate pSPpA and pSPGATA-4pA, respectively. Similarly, the dicistronic constructs used in the in vitro analyses were generated by amplifying a fragment spanning the Renilla ORF, the intercistronic region and the firefly ORF from pRF and subcloning this into pSP64 poly(A) to create cRPoly(A)30, cRGATA-4Fpoly(A)30 and cRpp27Fpoly(A)30 were created by amplification of the dicistronic regions of pRGATA-4F and pRpp27F (19), respectively, and subcloning into pSP64 poly(A) as before. The encephalomyocarditis virus (EMCV) IRES sequences were amplified from pIREShyg (Clontech) and inserted into the NcoI site of cRPoly(A)30. All constructs were sequenced to confirm their integrity and primer information is available upon request.

**In Vitro Translation of Mono- and Dicistronic mRNA Reporters**—The monocistronic and dicistronic plasmids were linearized and used as templates to in vitro transcribe the indicated RNAs using SP6 polymerase (19). These mRNAs were capped and subsequently used to program extracts of the indicated cell lines as described previously (19). The translation reactions were performed with the cap analog m7GpppG (Ambion) when indicated.

**Transient DNA and Dicistronic mRNA Transfections**—The indicated reporter constructs were transfected into cells using Lipofectamine Plus (Invitrogen) and normalized for transfection efficiency by co-transfection with pSVβGal (Promega). Cells were harvested 18 h following transfection and Renilla, firefly, and β-galactosidase activities were determined (Dual-Glo luciferase and β-galactosidase assay systems, Promega). RNA transfection was performed as previously described (26). Cells were harvested 12 h following RNA transfection, and luciferase activities determined. Relative luciferase values are expressed as activity per μg of protein.
RNAi Analysis—Knockdown experiments targeting the pRF transcript were accomplished based on the strategy of Van Eden et al. (28). A pool of double-stranded RNAs were synthesized (Dharmacon) targeting the Renilla luciferase ORF were annealed and co-transfected with the indicated mono or dicistrionic plasmids into HeLa cells. The targeting sequences within the Renilla ORF used were 5′/H11032-AAAGTTTATGATCCAGAA-CAA-3′ and 5′/H11032-AACAAAGGAAACGGATGATAA-3′.

Metabolic Labeling, Immunoprecipitation, and in Vitro PKC Activity Assays—P19CL6 or H9c2 cells were pulse-labeled with [35S]methionine/cystine at a final concentration of 100 Ci/ml. Cells were harvested following the indicated treatments and lysates prepared in ice-cold radioimmune precipitation assay buffer (100 g/ml cycloheximide at 4 °C). Following extraction with TRIzol, 3 µg of total RNA was loaded per lane in 1.4% formaldehyde-containing agarose gels, separated by electrophoresis and transferred to nylon membranes. Hybridization to riboprobes specific for the indicated transcripts was performed in ULTRAsyb hybridization buffer (Ambion). Blots were visualized by exposure to film or by a phosphorimager. RT-PCR was performed using the ImProm-II Reverse Transcription System (Promega) according to the instructions of the manufacturer. Polysome analysis was performed as previously described (11). Briefly, cells were lysed in buffer containing 100 µg/ml cycloheximide at 4 °C. Following removal of mitochondria and nuclei, supernatants were layered onto 15–50% sucrose gradients and spun at 38,000 rpm for 2 h at 4 °C in a SW 40 rotor (Beckman Instruments). Gradients were fractionated into eleven 1-ml fractions using an ISCO Density Gradient Fractionator at a flow rate of 3 ml/min.

GATA-4 protein was immunoprecipitated overnight at 4 °C with 1 µg of anti-GATA-4 antibody and then collected with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). The immunoprecipitate was washed four times in radioimmune precipitation assay buffer and fresh protease inhibitors and the complex pelleted for resuspension in SDS sample buffer. The samples were separated by SDS-PAGE, the gels dried and visualized using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Phosphor-densitometry was performed using ImageQuant (Molecular Dynamics) software. Collectively, immunoprecipitation controls included equal numbers of cells plated per flask and equal amounts of quantitated total protein lysate per sample with equivalent amounts of antibody. PKC activity was determined using the SigmaTECT® Protein Kinase C assay system (Promega, Madison, WI) as described by the manufacturer. Briefly, cell lysates were incubated with [γ-32P]ATP and PKC-biotinylated peptide substrate in substrate buffer at 30 °C for 5 min and subsequently spotted onto SAM biotin capture membrane (Promega). Membranes were washed and incorporated label measured by a scintillation counter.

RNA and Protein Analysis—Northern analysis was performed as previously described (19) with RNA purified from the indicated cell lines or in vitro translation reactions. Following extraction with TRIzol, 3 µg of total RNA was loaded per lane in 1.4% formaldehyde-containing agarose gels, separated by electrophoresis and transferred to nylon membranes. Hybridization to riboprobes specific for the indicated transcripts was performed in ULTRAhyb hybridization buffer (Ambion). Blots were visualized by exposure to film or by a phosphorimager. RT-PCR was performed using the ImProm-II Reverse Transcription System (Promega) according to the instructions of the manufacturer. Polysome analysis was performed as previously described (11). Briefly, cells were lysed in buffer containing 100 µg/ml cycloheximide at 4 °C. Following removal of mitochondria and nuclei, supernatants were layered onto 15–30% sucrose gradients and spun at 38,000 rpm for 2 h at 4 °C in a SW 40 rotor (Beckman Instruments). Gradients were fractionated into eleven 1-ml fractions using an ISCO Density Gradient Fractionator at a flow rate of 3 ml/min. The
profiles of the gradients were monitored via UV absorbance at 260 nm. RNAs from the individual fractions were pooled into a non-ribosomal and monosomal containing pool and a polysomal pool. These RNAs (100 ng) were subsequently used in real-time quantitative RT-PCR analyses of the indicated transcripts using the QuantiTect SYBR Green RT-PCR kit (Qiagen) in an Eppendorf Mastercycler equipped with a realplex2 optical module (Eppendorf AG, Germany). Transcript-specific oligonucleotides for the indicated mRNAs were designed to amplify 150-bp fragments. Immunoblots were performed using standard procedures. Briefly, cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na3VO4, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Samples were resolved on 4–20% SDS-PAGE gels, transferred to polyvinylidene difluoride (0.2 µm) membranes and probed with antibodies to the following proteins: phospho-eIF-2α and eIF-2α (Cell Signaling), Cdk1 (Abcam), BiP (Imgenex), actin (Sigma), GATA-4, MLC-2, and α-MHC antibodies were all from Santa Cruz Biotechnology.

RESULTS

The GATA-4 Leader Confers the Efficient Translation of Uncapped mRNA Reporters in Translationally Competent Cell Extracts—To begin to study the translational control of the human GATA-4 mRNA the transcriptional start site had to be determined and validated. We used 5′-RACE to isolate and characterize potential leaders. A single 5′-RACE product was amplified from total RNA extracted from either U87-MG or HeLa cells. The sequence was 518 nucleotides in length and was consistent with the previously published human 5′-UTR (21). The GATA-4 leader is 67% G-C rich and contains 18 upstream initiation codons, several of which are in inappropriate sequence context to support initiation by a scanning mechanism. Secondary structure prediction using the MFOLD algorithm of Zuker (29), predicted a highly stable structure with free energy values greater than −65 kcal/mol. We thus expected that the GATA-4 5′-UTR would be inhibitory to cap-dependent ribosomal scanning and may be translated in a cap-independent fashion. To determine if this was the case, we fused the GATA-4 5′-UTR immediately upstream of the firefly luciferase open reading frame in a SP6 promoter-based construct to generate pSPGATA-4pA as shown in Fig. 1A. In vitro transcribed mRNAs from the pSPGATA-4pA construct were either capped or not and used to program translation-competent cell-free extracts (30). As shown in Fig. 1B, uncapped pSPpA in vitro transcribed mRNA was poorly translated at a comparable concentration of the analog. Northern analysis showed that similar amounts of pSPpA and pSPGATA-4pA were translated in HeLa cells transfected with similar amounts of the respective plasmids. The results shown are the mean and S.D. from four independent transfections.
pSPGATA-4pA mRNAs of the expected size could be detected in the in vitro translation reactions (Fig. 1D). These data suggested that the GATA-4 5′-UTR sequences present in the pSPGATA-4pA construct do not affect steady-state mRNA levels, or are likely to contain splice sequences. Thus, the 5′-UTR of GATA-4 can confer efficient translation of uncapped reporter mRNAs.

**The Leader of the GATA-4 mRNA Enhances Translation in Vivo When Cap-dependent Translation Is Inhibited in cis**—Because we had previously observed that the GATA-4 mRNA was well translated under conditions of reduced eIF-4F complex formation (11), we sought to determine whether a similar affect could be seen if its translation were inhibited in cis. We introduced a stable hairpin structure (ΔG = −61 kcal/mol) (27) immediately distal to the SV40 promoter sequences in pGL to generate phpGL (Fig. 2A). This cis-acting structural element reduced the translation of the parental construct by 85% when transfected into HeLa cells (Fig. 2B). To assess whether the GATA-4 5′-UTR could mediate translation if cap-dependent translation was inhibited in this manner, we inserted the GATA-4 5′-UTR between the hairpin and the translation start site generating phpGATA-4L. This construct expressed 5-fold more luciferase activity in HeLa cell transfectants than the parental construct phpGL suggesting that the GATA-4 5′-UTR can promote translation initiation when cap-dependent scanning is reduced (Fig. 2B). To address whether cryptic promoters were present within the hairpin or GATA-4 5′-UTR sequences we removed the SV40 promoter sequences from phpGATA-4L to generate (p)phpGATA-4L and tested HeLa transfectants for luciferase activity. As can be seen (Fig. 2B), no significant luciferase activity was detected suggesting that these sequences, in the context of the monocistronic reporter plasmid, do not support promoter activity.

**Dicistronic Reporter mRNAs Containing the GATA-4 5′-UTR within the Intercistronic Region Demonstrate IRES Activity**—To determine whether the GATA-4 5′-UTR could function as an IRES in vivo, we introduced it into the intercistronic region within the dicistronic mRNA reporter plasmid pRF to generate pRGATA-4F (Fig. 3A). This plasmid contains the Renilla luciferase ORF as the first cistron and the firefly luciferase ORF as the second downstream cistron. In all the cell lines transfected with the pRGATA-4F construct downstream firefly luciferase activity was 6–10-fold higher as compared with values obtained for the control plasmid pRF (Fig. 3B). As a positive control, transfection with a construct baring the c-myc IRES in these lines also yielded firefly luciferase activities which were comparable to those obtained with pRGATA-4F. Renilla luciferase levels were comparable in all the cell lines tested, suggesting that the steady-state mRNA levels and the relative levels of...
GATA-4 mRNA IRES Activity

A schematic diagram of plasmids used in transient transfections. (p)RGATA-4F is identical to pRGATA-F other then removal of the SV40 promoter sequences. A stable hairpin structure was introduced upstream of the Renilla start to generate phpRGATA-4F. B, relative firefly and Renilla luciferase activities in extracts of the indicated transiently transfected cells with the indicated plasmids. pSV-β-gal was used as a control to normalize for transfection efficiencies. The relative firefly and Renilla luciferase conferred by the pRGATA-4F plasmid was normalized to 1 for each cell line. The results shown are the mean and S.D. from four independent transfections.

FIGURE 4. GATA-4 5'-UTR sequences do not facilitate ribosomal readthrough in dicistronic plasmids. A, schematic diagram of plasmids used in transient transfections. (p)RGATA-4F is identical to pRGATA-F other then removal of the SV40 promoter sequences. A stable hairpin structure was introduced upstream of the Renilla start to generate phpRGATA-4F. B, relative firefly and Renilla luciferase activities in extracts of the indicated transiently transfected cells with the indicated plasmids. pSV-β-gal was used as a control to normalize for transfection efficiencies. The relative firefly and Renilla luciferase conferred by the pRGATA-4F plasmid was normalized to 1 for each cell line. The results shown are the mean and S.D. from four independent transfections.

In an effort to more carefully examine the possibility of monocistronic RNAs containing the firefly luciferase ORF being generated in vivo from these dicistronic mRNAs, we transiently transfected our cultures with a dicistronic construct containing the firefly luciferase ORF between the indicated monocistronic regions (Fig. 2A). As shown in Fig. 3D, a single RNA species of the expected molecular mass was amplified from HeLa cells transfected with pRGATA-4F. No other RNAs were observed suggesting that no aberrant splicing was taking place. These results support the notion that the GATA-4 5'-UTR can initiate translation internally and the firefly luciferase activity observed is not likely based on cap-dependent initiation of shorter monocistronic reporter transcripts.

Ribosomal Readthrough Does Not Occur in Dicistronic Constructs Containing the GATA-4 5'-UTR—To determine if the GATA-4 5'-UTR could function in a dicistronic construct by facilitating termination codon readthrough of the Renilla ORF through the intercistronic region, we inserted the hairpin structure (Fig. 2A) upstream of the Renilla ORF within the dicistronic construct pRGATA-4F to generate phpRGATA-4F (Fig. 4A). If the GATA-4 5'-UTR sequences were mediating ribosomal readthrough of the Renilla termination codon, inhibiting translation of the upstream ORF should also result in a proportional reduction in firefly luciferase translation. We transfected either pRGATA-4F or phpRGATA-4F in the indicated cell lines and assessed the relative levels of luciferase activity in extracts prepared from these cells. As shown in Fig. 4B, introduction of the stable hairpin reduced Renilla luciferase activity by ~80% as compared with cells transfected with the control construct in the cell lines tested. However, the downstream firefly luciferase ORF remained well translated and luciferase activity did not change significantly comparing cells transfected with the hairpin-containing construct to those transfected with the control dicistronic construct lacking the hairpin structure (Fig. 4B). To control for the possibility of activating a cryptic promoter within the GATA-4 5'-UTR sequences in the context of the dicistronic reporter plasmid, we also inserted the GATA-4 5'-UTR sequences in a promoterless version of pRF and determined luciferase activity in HeLa cells transfected with this construct (p)RGATA-4F. As with the monocistronic promoterless control, (Fig. 2B) no significant luciferase activity was detected (Fig. 4B). These data support the notion that the GATA-4 5'-UTR does not stimulate readthrough of the Renilla termination codon.

Dicistronic RNA Transfections and Renilla Luciferase ORF Knockdown Demonstrate GATA-4 5'-UTR IRES Activity—Our data to this point indicated the presence of an IRES within the GATA-4 5'-UTR sequences. However, in the light of several recent reports (31–33) describing cryptic promoter activity or aberrant splicing events leading to the generation of monocistronic firefly ORF containing transcripts, we decided to transfect the in vitro transcribed, capped, and polyadenylated dicistronic RNAs indicated in Fig. 5A. This approach avoids the possibility of nuclear events altering reporter mRNA structure and requires only the cytoplasmic transfection of the transcripts. We generated plasmids, which produced the dicistronic RNAs, cRFpolyA30, cRgata-4polyA30, cRp27polyA30, and cRemcvFpolyA30 in vitro and transfected them into HeLa cells by liposomal encapsulation (26). Twelve hours following transfection, cell extracts were prepared, and luciferase activity determined. The data shown in Fig. 5B, the downstream cistron firefly luciferase activity was markedly higher (~10–12-fold increase) in extracts prepared from cells transfected with the dicistronic RNAs containing the cellular IRESs cRgata-4polyA30 and cRp27polyA30 mRNAs, and much higher in cells transfected with the dicistronic mRNA containing the viral (EMCV) IRES.
cRemcvFpolyA30 mRNA, as compared with the control RNA, cRFpolyA30.

In addition, we also attempted to knockdown Renilla luciferase expression by siRNA-mediated transfection. If downstream firefly luciferase expression was solely derived from dicistronic RNAs, not monocistronic firefly luciferase transcripts as a result of cryptic promoter activity or aberrant splicing events, one would expect that siRNAs targeting the coding region of the Renilla ORF should inhibit firefly expression proportionately (28, 31). Thus, siRNAs targeting the Renilla ORF were co-transfected into cells with the indicated constructs in Fig. 6. As can be seen, controls in which siRNAs were co-transfected with monocistronic Renilla and firefly luciferase constructs resulted in a specific reduction in Renilla luciferase activity while firefly activity was relatively unchanged. However, when the dicistronic control pRF was co-transfected with the siRNAs targeting the Renilla ORF, we observed a 90% reduction in Renilla activity concomitant with a reduction of firefly luciferase by a comparable amount, suggesting that both proteins are translated from the same dicistronic mRNA. Similarly, when cells were co-transfected with the siRNAs and either pRF containing the GATA-4 5'-UTR or the IRES sequences from the encephalomyocarditis virus within the intercistronic region, Renilla and firefly luciferase activities were reduced comparably. This suggested that the luciferase activities we observed were derived from dicistronic mRNAs and taken together, these data support the notion that the GATA-4 5'-UTR contains a bona fide IRES.

**GATA-4 IRES Activity Is Not Stimulated during Differentiation of Cardiomyocytes—**Because GATA-4 plays a prominent role in differentiation (34) and IRES activity has been reported to be stimulated during this process (22), we examined whether GATA-4 IRES activity was enhanced in P19CL6 cardiomyocytes induced to differentiate. P19CL6 clones which stably expressed the pRGATA-4F mRNA were initially examined for endogenous GATA-4 up-regulation as well as other proteins known to have increased expression following the induction of differentiation in this cell line. Shown in Fig. 7A, GATA-4 expression is increased by day 4 of culture in differentiation medium (DM) and is relatively high by day 12 in DM. Steady-state GATA-4 mRNA levels remained unchanged in differentiation media (Fig. 7B), supporting the notion that GATA-4 is regulated at the translational level. We then examined new GATA-4 protein synthesis in 35S-metabolically labeled P19CL6 cells during differentiation. As shown in Fig. 7C, new GATA-4 protein synthesis increased ~4-fold in cells in differentiation media by day 12 relative to cells maintained in growth medium. Increased expression of the cardiac contractile isoforms of myosin light and heavy chain proteins was also observed coincident with the induction of GATA-4 expression (Fig. 7A). The pRGATA-4F mRNA was also found to be full-length as determined by Northern analysis and was unaffected by culture in DM (not shown).

The phosphorylation of eIF-2α has been shown to be required for IRES activity under several different conditions including differentiation, although the underlying mechanisms are not clearly understood (15, 22, 54, 55). Thus, we determined the phosphorylation status of eIF-2α following the induction of differentiation in P19CL6 cells expressing pRGATA-4F mRNA. As shown in the inset of Fig. 7D, eIF-2α was significantly phosphorylated by 24 h after the induction of differen-
GATA-4 mRNA IRES Activity

A

|        | GM | DM |
|--------|----|----|
| cGATA-4 | 0  | 4  |
| MLC-2  | 0  | 4  |
| α-MHC  | 0  | 4  |
| actin  | 0  | 4  |

B

![Northern analysis of GATA-4 mRNA and 18S rRNA in P19CL6 cells transferred to differentiation medium for the indicated time points.](image)

C

![Relative fold change in firefly:Renilla ratio following the induction of differentiation in P19CL6 cardiomyocytes stably expressing pRGATA-4F.](image)

D

![Relative fold change in firefly:Renilla ratio following the induction of differentiation in P19CL6 cardiomyocytes stably expressing pRGATA-4F.](image)

FIGURE 7. GATA-4 IRES activity is not enhanced during differentiation. A, expression of GATA-4, MLC-2, α-MHC and actin in P19CL6 cardiomyocytes induced to differentiate (GM, growth media; DM, differentiation media). B, Northern analysis of GATA-4 mRNA and 18S rRNA in P19CL6 cells transferred to differentiation medium for the indicated time points. C, new GATA-4 protein synthesis in P19CL6 cells following transfer to differentiation media at the indicated time points. Fold increase in immunoprecipitated GATA-4 from P19CL6 cells in DM relative to GM is shown. Cells were pulsed with [35S]methionine/cystine during the last 3 h of culture in either DM or GM. Data presented are the mean and S.D. of three independent experiments. D, relative fold change in firefly:Renilla ratio following the induction of differentiation in P19CL6 cardiomyocytes stably expressing pRGATA-4F. The basal level ratio of firefly:Renilla was normalized to 1 prior to the addition of differentiation medium. Circles denote values obtained from cells expressing the pRGATA-4F mRNA, while triangles denote values obtained from cells expressing the control prF mRNA in growth medium (open) and differentiation medium (closed). Three independent stable transformants were tested, and the mean and S.D. luciferase activities are shown. Inset, immunoblot analysis of eIF-2α phosphorylation on Ser-51 and total eIF-2α levels following the induction of differentiation in P19CL6 cardiomyocytes.

tiation and was only slightly detectable by 48 h. We then examined luciferase activity in lysates from cells induced to differentiate at various time points as compared with cultures maintained in growth medium. As shown in Fig. 7D, there was no significant increase in the ratio of firefly:Renilla luciferase activities in cells expressing the pRGATA-4F mRNA at any time following the induction of differentiation. Similarly, in cells stably expressing the control prF mRNA, no changes in firefly:Renilla luciferase ratios were observed during differentiation. These data suggested that IRES-mediated initiation of GATA-4 mRNA does not appear to occur in differentiating P19CL6 cardiomyocytes.

GATA-4 IRES Activity Is Stimulated during Vasopressin-induced Hypertrophy of Cardiomyocytes—Because many IRESs are most active during times of cellular stress and when general mRNA translation initiation is inhibited (13, 35), we examined whether GATA-4 IRES activity was stimulated following the induction of hypertrophy in cardiac myocytes. We initially examined GATA-4 expression in H9c2 stimulated to undergo hypertrophy by vasopressin exposure (36). Treatment of H9c2 with vasopressin at concentrations of 10 nm or greater and at physiologic extracellular Ca\(^{2+}\) concentrations is biphasic and is known to result in a rapid suppression of overall protein synthesis in association with phosphorylation of eIF-2α reaching maximum inhibition by 30 min of exposure to the hormone. This initial reduction in protein synthesis is followed by a 1.2–1.5-fold stimulation of protein synthesis by 12–16 h of exposure (37). Significant hypertrophic growth occurs by 24 h following vasopressin exposure with no change in cell number or cell cycle distribution (36, 37). Thus, we examined the affects of vasopressin on GATA-4 expression at early and later time points. As shown in Fig. 8A, treatment of H9c2 cells with vasopressin resulted in a ~10–12-fold increase in GATA-4 protein levels as early as 30 min following exposure to the hormone. The molecular chaperone BiP, a protein whose expression is known to be stimulated following vasopressin-induced hypertrophy (38, 39), demonstrated increasing expression, while Cdk1 protein content was inhibited within 1 h of exposure consistent with hypertrophic reprogramming. Northern analysis of steady-state GATA-4 mRNA abundance demonstrated only a ~2-fold increase in transcript levels (Fig. 8B) by 120 min following vasopressin. While these data were suggestive of translational control of the GATA-4 mRNA following vasopressin treatment, we examined new GATA-4 protein synthesis following exposure to the hormone. As shown in Fig. 8C, new GATA-4 protein synthesis was stimulated ~7-fold relative to levels in unstimulated control cells within 30 min of exposure. Increased synthesis was then maintained at ~4–5-fold over unstimulated levels during hypertrophic growth. Polysomal analysis of GATA-4 mRNA also demonstrated increased polysomal association of GATA-4 mRNA by 30 min of vasopressin treatment (Fig. 8D) with 51% of the mRNA found in polysomal fractions as compared with 12% at time 0 of vasopressin treatment. A determination of the steady-state mRNA levels by summing the nonribosomal, monosomal, and polysomal signals also showed a ~2.5-fold increase in mRNA abundance of GATA-4 mRNA following vasopressin exposure (not shown). To determine whether GATA-4 IRES-mediated translation initiation was also increased following vasopressin exposure, we transiently transfected H9c2 cells with pRGATA-4F and exposed transfectants to vasopressin and assessed luciferase activities at various time points following exposure. As shown in Fig. 8E, firefly luciferase activity was significantly increased within 30 min of vasopressin exposure and maintained high levels of activity consistent with the increases seen in new GATA-4 synthesis, translational efficiency and total GATA-4 protein expression following vasopressin treatment. No significant change in the firefly:Renilla ratios
was observed in control cells transfected with the pRF plasmid following exposure to vasopressin. GATA-4 IRES activity remained significantly higher, ~50% of the maximal value during the hypertrophic growth phase following hormone stimulation. These experiments suggested that GATA-4 IRES activity was stimulated during vasopressin-induced hypertrophic growth of H9c2 cardiomyocytes.

Protein Kinase C Activity Is Required for GATA-4 IRES Activity during Hypertrophy—Several reports have implicated PKC activity in the induction of translation of specific mRNAs following vasopressin treatment in H9c2 cardiomyocytes (37, 38). Thus, we examined whether PKC activity was required for GATA-4 IRES activity during the hypertrophic response to vasopressin. To inhibit PKC activity we utilized a cell-permeable myristoylated pseudosubstrate from PKC, inhibitor 20–28 (37, 40). As shown in Fig. 9A, H9c2 cells exposed to vasopressin displayed marked activation of PKC within 30 min of exposure. However, PKC activity was inhibited (~95%) by the pseudosubstrate peptide at a concentration of 12 μM. H9c2 cells were transiently transfected with pRGATA-4F and treated with vasopressin in the presence or absence of inhibitor 20–28 peptide. As shown in Fig. 9B, the ratio of firefly to Renilla luciferase activity increased ~9-fold within 30 min of vasopressin exposure in the absence of inhibitor 20–28 peptide. This relative increase in firefly luciferase activity subsequently diminished by ~50% of maximal values coincident with the dephosphorylation of eIF-2α (Fig. 9C) and the induction of cap-dependent protein synthesis by 1 h following exposure to vasopressin. We note however, that the observed decrease in GATA-4 IRES activity from its maximal activity at 30 min following vasopressin exposure to its submaxi-
mal value would reflect decay of the firefly luciferase reporter. This apparent decrease in luciferase activity is consistent with its relatively short protein half-life in vivo (~2 h) (41). The ratio of firefly to Renilla luciferase activity only minimally increased following exposure to vasopressin in cells pretreated with the PKC pseudosubstrate peptide. These results suggested that PKC activity was required for the induction of GATA-4 IRES activity following vasopressin exposure. The GATA-4 increase in protein abundance following vasopressin exposure was also inhibited by treatment with the PKC inhibitor (Fig. 9D). Similarly, BiP expression was also dependent on PKC activity following vasopressin exposure as had been demonstrated previously (38).

**Supra-physiological Ca^{2+}** Levels Reduce Vasopressin-induced GATA-4 IRES Activity—Because the continued synthesis of several proteins has been demonstrated to be sensitive to ER-associated Ca^{2+} mobilization in H9c2 cells following vasopressin-induced hypertrophy (37, 39), we tested whether elevated extracellular Ca^{2+} levels affected GATA-4 IRES activity and protein levels following vasopressin exposure. These high Ca^{2+} levels are expected to diminish ER Ca^{2+} depletion following hormonal challenge (37). As shown in Fig. 9B, treatment of cells transfected with pRGATA-4F with vasopressin in the presence of 3 mM Ca^{2+} resulted in an ~90% reduction in firefly:Renilla luciferase activity ratio by 30 min as compared with controls containing physiological Ca^{2+} levels. Vasopressin-mediated induction of GATA-4 protein levels were also significantly reduced in the presence of elevated Ca^{2+} (Fig. 9C). In contrast, when we pretreated cells with the Ca^{2+} channel blocker nifedipine, which blocks plasmalemmal Ca^{2+} entry and has been previously demonstrated to reduce Ca^{2+} content of H9c2 cells by ~40% at the concentration used (37), vasopressin treatment still resulted in the comparable stimulation of GATA-4 IRES activity (Fig. 9B) and induction of protein levels (Fig. 9D) as had previously been observed with the hormone alone. These data support the notion that the increased IRES activity and protein expression of GATA-4 in H9c2 cells following vasopressin treatment is dependent on the mobilization of Ca^{2+} sequestered by the ER.

**FIGURE 9.** Vasopressin-induced GATA-4 IRES activity requires PKC and is inhibited by supraphysiological Ca^{2+} levels. A, AVP-induced PKC activity is inhibited by a pseudosubstrate peptide inhibitor 20–28 (PS). H9c2 cells were exposed to AVP for 30 min in the absence or presence of the PKC inhibitor at the indicated concentrations. The results shown are the mean and S.D. of three independent experiments. B, H9c2 cells were transiently transfected with pRGATA-4F and exposed to AVP alone or with indicated inhibitors or medium containing elevated Ca^{2+} for the indicated time points. The relative fold change in firefly:Renilla ratio is shown obtained following the indicated treatments as compared with luciferase values obtained with no treatments. The results shown are the mean and S.D. of four individual experiments. C, kinetics of eIF-2α phosphorylation in H9c2 cells following exposure to AVP for the indicated time points. Immunoblot was probed with an antibody specific for serine 51 phosphorylated eIF2α and total eIF2α. D, steady-state expression levels of GATA-4, BiP, Cdk1, and actin in H9c2 cells under the conditions as in B. Immunoblots in C and D are representative of four independent experiments with similar results. E, transradial regulation of the GATA-4 IRES may involve a PKC-induced or activated ITAF following binding of vasopressin to V1 receptors (V1R) and subsequent activation of PKC.
DISCUSSION

Our previous studies implicated a cap-independent mechanism of translation initiation for a number of specific transcripts under conditions of impaired eIF-4F formation (11). In this report we have investigated the mechanism of initiation of the GATA-4 mRNA and identified an IRES within its 5′-UTR. Moreover, we have confirmed IRES activity by several criteria, which support the hypothesis that the GATA-4 transcript is able to initiate translation internally such that cap recognition is not required. We also demonstrate that the GATA-4 mRNA is subject to translational control during differentiation and hypertrophy. We have also demonstrated that GATA-4 IRES activity is stimulated maximally, coincident with phosphorylation of eIF-2α, and continues during hypertrophic growth of cardiomyocytes following treatment with the hormone arginine vasopressin in a PKC-dependent manner. Additionally, our results suggest that Ca2+/calmodulin release from the sarcoplasmic/endoplasmic reticulum (S(E)R) and activation of PKC. The subsequent recovery from this inhibition results in an increase in cap-dependent protein synthesis, during the hypertrophic growth phase, at which time GATA-4 IRES activity is maintained at submaximal levels and is dependent on PKC activity for continued function. Thus, GATA-4 protein synthesis is specifically induced during hypertrophic reprogramming and its synthesis is maintained during hypertrophy leading to transactivation of genes required for the accumulation of cell size.

The observation that Ca2+ release from S(E)R stores induces rapid GATA-4 IRES activity is intriguing. Several Ca2+-binding proteins are known to interact with RNAs to regulate mRNA stability (42, 43) and indeed we noted a small but significant increase in the steady-state mRNA levels of GATA-4 following vasopressin exposure. This increase in abundance may be the result of effects on transcription or mRNA stability. However, it is also possible that such Ca2+-binding factors may directly or indirectly regulate IRES activity in response to changes in cytosolic Ca2+ levels. Indeed, the IRES-trans-acting factors (ITAFs) hnRNP A and hnRNP C are known to bind calmodulin in the presence of Ca2+ (44) and in addition, hnRNP A is also known to be regulated by PKC phosphorylation which alters its ability to bind RNA (45, 46). Thus, these data potentially link Ca2+ signaling pathways to ITAF activity. Ongoing studies will investigate whether hnRNPA regulates GATA-4 IRES activity because our experiments show a requirement for PKC activity for GATA-4 IRES function during hypertrophy.

Our data imply that the GATA-4 IRES is also active during hypertrophic growth when the default cap-dependent initiation mechanism is active. Several reports have documented the requirement for mTOR and its downstream effector S6K1 in cardiac myocyte hypertrophy during the activation of cap-dependent initiation (47–51). Our data support a role for the cap-independent synthesis of proteins during this process. The activation of cap-dependent initiation may seem incompatible with the continued IRES-mediated initiation of GATA-4 mRNA we observed in our experiments. However, current models regarding the control of cap-independent protein synthesis suggest that some IRES-containing mRNAs may be able to initiate translation by both cap-dependent and cap-independent mechanisms depending on ensuing conditions and that the two mechanisms may not be mutually exclusive (13, 15). Indeed, several IRES have strict requirements for canonical initiation factors (15, 52). Furthermore, a cap-dependent IRES has been described within the eIF-4G1 mRNA which is active following poliovirus infection (53). We speculate that during cardiomyocyte hypertrophic reprogramming a specific ITAF(s) is activated which enhances GATA-4 IRES activity and allows continued, albeit, reduced activity while conditions for cap-dependent initiation are also permissive.

Several studies have demonstrated a role for eIF-2α phosphorylation in the regulation of particular IRESs (22, 54, 55). However, the mechanisms by which enhanced eIF-2α phosphorylation leads to stimulation of IRES activity is not well understood. One possible mechanism may be the ability of a particular IRES to direct efficient initiation in the absence of translation-competent ternary complexes (eIF2/GTP/tRNA^Met^) as a result of eIF-2α phosphorylation. Recent reports have described initiation factor-independent translation for viral IRESs (56, 57). Whether any cellular IRESs are able to initiate in a similar manner is unknown. Another possibility may be that eIF-2α phosphorylation indirectly stimulates IRES activity via induction or activation of an ITAF. During vasopressin-induced hypertrophy, stimulation of GATA-4 IRES activity correlated with enhanced eIF-2α phosphorylation (Fig. 9, B and C), however, the mechanism by which these two events may be linked is unclear. It is possible that eIF-2α phosphorylation results in accumulation or activation of an ITAF, which is required for GATA-4 IRES activity as has been postulated for other cellular IRESs whose activity is regulated by eIF-2α phosphorylation status (54). However, the observation that the GATA-4 IRES did not show elevated activity during P19CL6 cell differentiation, even though increased phosphorylation was observed, suggests that eIF-2α phosphorylation alone is insufficient to stimulate GATA-4 IRES activity directly. Specific ITAFs required for GATA-4 IRES activity may not be present or activated such as to stimulate IRES activity under this condition. In fact, we examined whether PKC activity was stimulated during differentiation of P19CL6 cells and did not observe significant activation (not shown). Thus, it is possible that a PKC-induced or activated ITAF regulates GATA-4 IRES activity during vasopressin-induced hypertrophy which is absent during differentiation (Fig. 9E). Future experiments will address what additional factors are required for GATA-4 IRES activity under these conditions.

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