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Myocyte-Specific M-CAT and MEF-1 Elements Regulate G-Protein Gamma 3 Gene (γ_3) Expression in Cardiac Myocytes

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Little is known regarding the mechanisms that control the expression of G-protein α, β, and γ subtypes. We have previously shown that the G-protein γ_3 gene is expressed in the heart, brain, lung, spleen, kidney, muscle, and testis in mice. We have also reported that the G-protein γ_3 subunit is expressed in rat cardiac myocytes, but not in cardiac fibroblasts. Other studies have shown that the γ_3 subunit couples to the angiotensin A1A receptor in portal vein myocytes, and has been shown to mediate β-adrenergic desensitization in cardiac myocytes treated with atorvastatin. In the present study, we evaluated G-protein γ_3 promoter-luciferase reporter constructs in primary myocytes to identify key regulatory promoter regions. We identified two important regions of the promoter (upstream promoter region [UPR] and downstream promoter region [DPR]), which are required for expression in cardiac myocytes. We observed that removal of 48 bp in the UPR diminished gene transcription by 75%, and that the UPR contains consensus elements for myocyte-specific M-CAT and myocyte enhancer factor 1 (MEF-1) elements. The UPR and DPR share transcription factor elements for myocyte-specific M-CAT element. We observed that cardiac myocyte proteins bind to γ_3 oligonucleotides containing transcription factor elements for myocyte-specific M-CAT and MEF-1. Myocyte-specific M-CAT proteins were supershifted with transcriptional enhancer factor-1 (TEF-1) antibodies binding to the γ_3 M-CAT element, which is in agreement with reports showing that the M-CAT element binds the TEF-1 family of transcription factors. The 150 bp DPR contains three M-CAT elements, an INR element, an upstream stimulatory factor 1 element, and the transcription start site. We have shown that myocyte γ_3 gene expression is regulated by myocyte-specific M-CAT and MEF-1 elements.

Introduction

A family of heterotrimeric G-proteins transmits signals from heptahelical receptors to their respective effectors (Gilman, 1987; Neer, 1995). Relatively little is known about G-protein βγ subunits in the heart despite mounting evidence demonstrating their importance in receptor recognition (Gilman, 1987; Birnbaumer, 1992; Neer, 1995) and effector regulation (Kleuss *et al*., 1993; Wickman *et al*., 1994). The effector targets of G-protein βγ subunits include ion channels, phospholipase A2, phospholipase C, adenylyl cyclase, G-protein–coupled receptor kinases, PI3 kinase, Ras, Raf-1, Bruton tyrosine kinase, Tsk tyrosine kinase, and plasma membrane calcium pumps (Clapham and Neer, 1997). Both reconstitution and genetic approaches have shown that the nature of the γ subunit is an important determinant for interaction of the G-protein with the receptor (Kleuss *et al*., 1993; Richardson and Robishaw, 1999). Studies have revealed that the γ_3 subunit couples to the somatostatin receptor in the brain (Kleuss *et al*., 1993), and the fluorescent protein-tagged γ_3 subunit regulated Ca^{2+} channel inactivation (Zhou *et al*., 2003). Mice lacking the γ_3 gene showed susceptibility to seizures and a defective immune response (Schwindinger *et al*., 2004; Dubeykovskiy *et al*., 2007). In the heart, studies have shown that the γ_3 subunit couples the angiotensin AT1A receptor to increase cytoplasmic Ca^{+2} in rat portal vein myocytes (Macrez *et al*., 1997), and the overexpression of the γ_3 subunit resulted in severe heart defects and upregulation of mitogen-activated protein kinase activity in zebrafish (Bell *et al*., 1999; Kelly *et al*., 2001). Other investigators have shown that β-adrenergic desensitization with atorvastatin involves reduced isoprenylation of γ_3 proteins in cardiac myocytes (Muhlhauser *et al*., 2006). We have previously demonstrated that the γ_3 gene is expressed in cardiac myocytes, but not in fibroblasts (Hansen *et al*., 1995). To investigate the regulation of the γ_3 subunit in the heart, we have evaluated the expression levels of γ_3 luciferase reporter constructs in cardiac myocytes.

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Materials and Methods

Cardiac myocytes

Cardiac myocytes were prepared from the hearts of 1–2-day-old Sprague-Dawley rats (Charles River Company, Wilmington, MD) as reported (McWhinney *et al*., 1997; McWhinney *et al*., 2000a,b). Briefly, the ventricles were removed and digested with a mixture of trypsin, chymotrypsin, and elastase in a Celstir apparatus at 37°C. Myocytes were suspended in DMEM containing 10% fetal bovine serum, 100 μM 5-bromo-2′-deoxyuridine, 50 units/mL penicillin, and 50 μg/mL streptomycin and plated at a density of 10×10^6/100-mm dish on 0.1% gelatin-coated tissue culture dishes. After 24 h, the cardiac myocytes were serum-starved in DMEM with 0.1% fetal bovine serum for 72 h prior to experiments.

Promoter-luciferase constructs

The p(*Eco*RI)-γ_3-luciferase-reporter contains DNA from *Eco*RI to *Kpn*I restriction enzyme sites (Dubeykovskiy *et al*., 2007), and was cloned into the *Kpn*I site in the p-GL3-basic luciferase vector (Promega, Madison, WI). The p(*Sac*I)-γ_3-luciferase reporter was generated by using restriction enzymes *Sac*I (−1550) and *Kpn*I (−555). The *Sac*I-*Kpn*I fragment was subcloned into the pGL3-basic vector. The p(*Fsp*I)-γ_3-luciferase reporter was generated using restriction enzymes *Fsp*I (−1502) and *Kpn*I. The *Fsp*I/*Kpn*I fragment was subcloned into the *Sma*I site in the pGL3-basic vector. The p(*Bsp*mI)-γ_3-luciferase reporter was generated using restriction enzymes *Sac*I and *Bsp*mI to remove it from the p(*Eco*RI)-γ_3-luciferase reporter. The α-myosin heavy chain (α-MHC) promoter was removed with restriction enzyme digestion with *Hin*dIII (Gupta *et al*., 1994). The 4.2 kb α-MHC promoter was subcloned into the *Hin*dIII site of the basic pGL3-luciferase construct (Promega).

Transfection of cells

Cultured cells and/or cardiac myocytes were transfected with 1 μg of plasmid DNA. An aliquot of 1 μg of luciferase reporter plasmid was mixed with 7 μL of Lipofect-Amine Plus reagent (Invitrogen, Carlsbad, CA) and 100 μL of Opti-MEM medium (Invitrogen). After 5 h, the transfection medium was removed and the cells were maintained in Opti-MEM medium without antibiotics. Posttransfection 24 h, cells were transferred to DMEM medium (Invitrogen) with 10% fetal calf serum and antibiotics for 72 h (McWhinney *et al*., 2000a,b).

Luciferase gene expression

After 72 h of transfection, the cells were disrupted with 1× lysis buffer (Promega). Cell lysates were assayed for luciferase gene expression using a Bertholt luminometer and Promega substrate reagents (McWhinney *et al*., 2000a,b). For luciferase gene expression assays, the cells were lysed in 0.5 mL of 1× lysis buffer (Promega). The cell lysates were centrifuged at 15,000 *g* for 1 min at 4°C, and the supernatants were transferred to a new tube. Approximately 50 μL of the supernatant was assayed in triplicate for luciferase gene expression using a luciferase assay system with reporter lysis buffer (Promega). Relative light units were measured utilizing a Lumat LB9507 EG&G Berthold luminometer with cell lysates. Following luciferase assays, β-galactosidase enzyme assays were performed in triplicate samples. The cell lysates from the luciferase assays were assayed spectrophotometrically for β-galactosidase enzyme activity using the β-galactosidase enzyme assay system with reporter lysis buffer (Promega). The luciferase relative light unit numbers were divided by the β-galactosidase enzyme assay numbers to correct for transfection efficiency in cells. The luciferase activities were normalized to β-galactosidase enzyme activities to correct for differences in transfection efficiency, and the normalized luciferase activities were expressed as fold induction relative to the pGL3-basic luciferase reporters with no promoter (control). Both γ_3-luciferase gene expression and α-MHC-pGL3-luciferase expression (Gupta *et al*., 1994) were measured in transiently transfected cardiac myocytes and/or cell lines (McWhinney *et al*., 2000a,b). Each experiment was repeated at least three times.

Electrophoretic mobility shift analysis

Nuclear and/or whole-cell extracts were used in electrophoretic mobility shift analysis (EMSA) and were incubated for 20 min at 25°C with double-stranded oligonucleotides (McWhinney, 1996; McWhinney *et al*., 2000a,b). Protein extracts were prepared from cultured cells as reported (McWhinney, 1996; McWhinney *et al*., 1997). Competition experiments were performed with unlabeled oligonucleotides utilizing a 100-fold excess of competitor oligonucleotides to assess the specificity of the protein–DNA interactions in the EMSA. Protein complexes were electrophoresed on a 4.5% native polyacrylamide gel in 1× tris-glycine buffer (Sigma-Aldrich, Inc., St. Louis, MO) as reported (McWhinney *et al*., 2000a,b). Quantitations were made on a PhosphoImager (Model SF; Molecular Dynamics, GE Healthcare, Piscataway, NJ) using ImageQuant Program.

Protein extracts

Protein extracts were prepared from cultured cardiac myocytes using modified protocols for whole-cell extracts and nuclear extracts as reported (McWhinney *et al*., 1997). Briefly, cells were removed from tissue culture plates using Versene (phosphate-buffered saline with 0.2g/L EDTA; Invitrogen) and/or 0.25% trypsin (Invitrogen). Cells were washed in phosphate-buffered saline (9.0 g/L NaCl, 0.144 g/L KH_2PO_4, and 0.795 g/L NA_2HPO_4 $7H_2O$; Invitrogen) and then with buffer A (20 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.2 mM EDTA). Cells were resuspended in buffer A with 0.625% NP40 detergent and allowed to swell on ice for 15 min. Cell suspension was dounced 10 times to lyse cells, and nuclei were pelleted at 14,000 rpm for 30 s at 4°C. The pelleted nuclei were resuspended in 50 μL of buffer C (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 375 mM KCl), and incubated on ice for 20 min to extract proteins. The sample was centrifuged for 5 min at 14,000 rpm at 4°C to pellet nuclear membranes. The supernatant was aliquoted and stored at −80°C.

Oligonucleotides for EMSA

The oligonucleotides were end-labeled with T_4 polynucleotide kinase (Promega) and ^{32}P-γ-ATP (Dupont, NEN, Boston, MA) utilizing 10× kinase buffer (Promega) for 15 min at 37°C (McWhinney, 1996). The unincorporated nucleotides were

removed by filtration with MW 3000 membranes (Amicon, Beverly, MA). The oligonucleotides were resuspended in 100 μL of TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0). Listed below are the γ_3 DNA sequences from the 1.0 kb promoter for the double-stranded oligonucleotides used in EMSA experiments. The identified transcription factor elements are underlined.

Upstream promoter region (UPR) oligonucleotides:
Myocyte enhancer factor 1 (MEF-1):
5′-GAGCCTTGCACCCTGCCAG-3′
M-CAT: 5′-TGCCAGAATCTGAATCTTGTG-3′

Downstream promoter region (DPR) oligonucleotides:
M-CAT/DPR (+C/D):
5′-GTGTCGCTTGGGGATGCGAC-3′
M-CAT/R (+C/R):
5′-CCAGGCGGTTGGTATGCGCCAGAATC-3′
M-CAT/E: 5′-CTGTCCAGGCAGTTGGTATGCGCC-3′

Upstream stimulatory factor (USF):
5′-CCGGAGGCTGACTCCCTTGCTGTC-3′

Antibodies

Antibodies were purchased from Santa Cruz Biochemicals (USF-1 sc-229x [c-20]x and MyoD sc-760x [M-318]x), and were used in EMSA experiments.

Cell lines

The HL-1 myocyte cell line was obtained from Proctor and Gamble Company (Cincinnati, OH) and Dr. William Claycomb (McWhinney *et al*., 2000b). Briefly, the HL-1 cells were maintained in Ex-cell 320 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Invitrogen), 10 μg/mL endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 1 μM retinoic acid (Sigma), 10 μM norepinephrine (Invitrogen), 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and an additional 1× nonessential amino acids (Invitrogen). The HL-1 cells were plated at a density of 8.0×10^4 cells/cm^2 on dishes precoated with 0.1% gelatin and 0.1% fibronectin on 100-mm dishes (10^6 cells). The cells were switched to medium without norepinephrine for 4 days prior to experiments.

PCR

Cloned γ_3 DNA was used as templates for the following primers to generate the 150-bp γ_3 DPR fragment (McWhinney *et al*., 2000b) using the Advantage Plus PCR kit (Clontech, Palo Alto, CA). Cycling: 25 cycles of 94°C 30 s, 68°C 2 min, final 68°C for 5 min, PTC-100 Thermal Controller (MJ Research, Watertown, MA). PCR products or oligonucleotides were end-labeled with T4 polynucleotide kinase (Promega) and ^{32}-P-γ-ATP (Dupont) utilizing 10× kinase buffer (Promega) for 15 min at 25°C. Sense oligonucleotide: 5′-GCAGGTAGTGA GAGAGCAGC-3′; antisense oligonucleotide: 5′-CTGGTACC TGCTGAAGGATGC-3′.

Results

Luciferase γ_3 expression

We have previously shown that the γ_3 gene is expressed in the heart, brain, lung, spleen, kidney, muscle, and testis in mice (Schwindinger *et al*., 2004; Dubeykovskiy *et al*., 2007), and in rat that the γ_3 subunit is expressed in cardiac myocytes, but not cardiac fibroblasts (Hansen *et al*., 1995). To understand the regulation of the γ_3 promoter in the heart, we analyzed the ability of the γ_3 promoter to drive luciferase gene transcription in cardiac myocytes. We prepared a limited series of γ_3 promoter luciferase reporters and evaluated their effectiveness in transfected cardiac myocytes (McWhinney *et al*., 2000b), an HL-1 myocyte cell line (McWhinney *et al*., 2000b), and a Swiss 3T3 fibroblast cell line (Kurokawa *et al*., 1994). The experiments in fibroblasts were used as negative controls. To assess promoter activity, we inserted 7.1 kb of γ_3 DNA upstream of the gene encoding firefly luciferase gene in the pGL-3-basic-luciferase reporter construct generating the p(*Eco*RI)-γ_3-luciferase reporter construct (Fig. 1A). The promoter activity of this construct was determined by transient transfection expression in cardiac myocytes (McWhinney *et al*., 2000a), myocyte HL-1 cells (McWhinney *et al*., 2000b), and Swiss 3T3 fibroblasts (Kurokawa *et al*., 1994). The luciferase activities were normalized to β-galactosidase enzyme activities to correct for differences in transfection efficiency, and the normalized luciferase activities were expressed as fold induction relative to the pGL3-basic luciferase reporters with no promoter (control). We demonstrate that the p(*Eco*RI)-γ_3-luciferase reporter has 2.4-fold activity in cardiac myocytes and 3.3-fold activity in myocyte HL-1 cells, while displaying no activity in fibroblasts compared to vector (Fig. 1A). Thus, the γ_3 luciferase reporter construct shows activity only in the cell types previously reported to express γ_3 mRNA and does not show activity in cell types reported not to express γ_3 mRNA (Hansen *et al*., 1995; Downes *et al*., 1998).

Promoter analysis

To map regulatory elements in the γ_3 promoter region, we generated luciferase reporters with varying amounts of γ_3 promoter from the p(*Eco*RI)-γ_3-luciferase reporter construct. We analyzed the different γ_3 luciferase reporters in transfected cardiac rat myocytes and fibroblasts (Fig. 1B). Fibroblasts are used as negative controls for the luciferase assays. The p(*Sac*I)-γ_3-luciferase construct showed enhanced 4.8-fold luciferase expression in cardiac myocytes with only control-level activity in fibroblasts (Fig. 1B). These data show that the 1.0 kb p(*Sac*I)-γ_3-luciferase construct is expressed at two-fold higher levels in cardiac myocytes than the original p(*Eco*RI)-γ_3-luciferase construct, demonstrating that the 7.1 kb p(*Eco*RI)-γ_3-luciferase construct contains negative regulatory elements. The p(*Fsp*I)-γ_3-luciferase reporter construct, which has 48 bp removed from the 5′ end of the construct, demonstrated a 3.6-fold decrease in luciferase activity, which is 75% of the promoter activity in cardiac myocytes (Fig. 1B). The p(*Bsp*mI)-γ_3-luciferase reporter construct, which has 150 bp removed between the *Bsp*MI and *Kpn*I restriction enzyme sites at the 3′ end of the promoter, displayed a 4.8-fold decrease to control levels in cardiac myocytes. Neither the p(*Fsp*I)-γ_3-luciferase reporter construct nor the p(*Bsp*MI)-γ_3-luciferase reporter construct showed any activity in fibroblasts, which were used as negative controls (data not shown). These data demonstrate that both the 48 bp (UPR) and 150 bp (DPR) promoter sequences contain important regulatory elements for expression in cardiac myocytes.

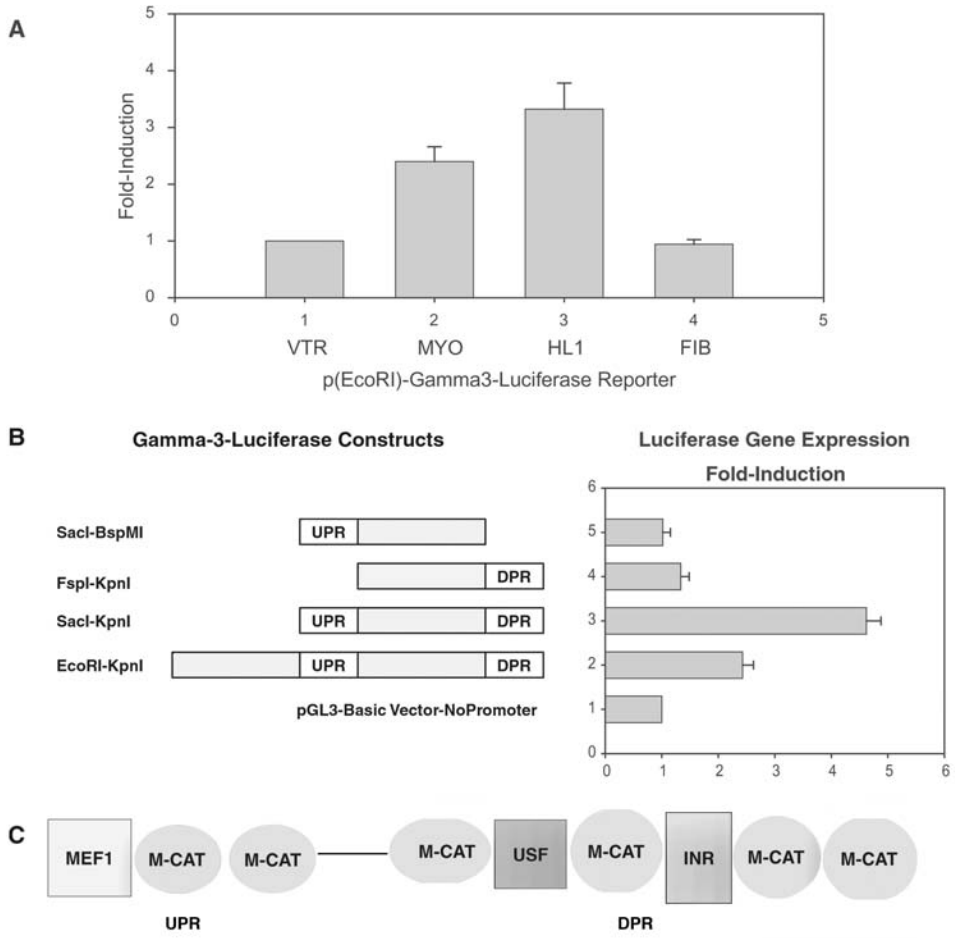

FIG. 1. Luciferase expression. Luciferase gene expression was measured in transfected cells. Cell lysates were isolated at 72-h posttransfection and assayed for luciferase gene expression. The reported fold induction is the relative change in luciferase reporter gene expression between the transfected cells with the pGL3-basic vector and different γ_3-luciferase constructs ($n = 5$). (**A**) The p(*Eco*RI)-luciferase expression was measured in transfected cardiac myocytes (MYO), HL-1 myocyte cell line (HL1), and Swiss 3T3 fibroblast cell lines (FIB) as fold induction over vector (VTR). (**B**) γ_3-Luciferase reporters in cardiac myocytes and fibroblasts. (**C**) UPR and DPR schematic.

Transcription factor promoter elements

The 1.0 kb p(*Sac*I)-γ_3-luciferase construct demonstrated the highest luciferase activity, showing that it contains important regulatory regions for expression in cardiac myocytes. Since removal of 48 bp from the p(*Sac*I)-γ_3-luciferase construct reduced gene transcription by 75% in cardiac myocytes, we created an oligonucleotide that covers the 48 bp UPR to determine if myocyte proteins would bind to this DNA.

To evaluate protein binding to individual transcription factor elements within the UPR, we synthesized two oligonucleotides, which cover 71% of the UPR, excluding a TG repetitive element at the end of the 48 bp region (Fig. 1C). In Figure 2A, we observed that proteins bind to the M-CAT oligonucleotides forming one protein complex. We demonstrate that a 100-fold excess of unlabeled self M-CAT oligonucleotides competed the protein complex, but that a nonspecific (+NS) oligonucleotide had no effect on the myocyte protein complex (Fig. 2A). These data show that the complex is specific for the M-CAT element. In addition, cardiac myocyte proteins binding to the M-CAT oligonucleotide were supershifted with transcriptional enhancer factor-1 (TEF-1) antibodies (Fig. 2B). The M-CAT element is reported to bind the TEF-1 family of transcription factors (Stewart *et al.*, 1998).

We noticed that the UPR contained a 14 bp consensus element for myocyte-specific MEF-1 (Buskin and Hauschka, 1989; Horlick and Benfield, 1989; Amacher *et al.*, 1993; Apone and Hauschka, 1995; Feo *et al.*, 1995; Klamut *et al.*, 1997), which is adjacent to two myocyte-specific M-CAT elements (Berberich *et al.*, 1993; Gupta *et al.*, 1994). In Figure 2C, we observed that proteins bind to the MEF-1 element forming a single complex. To investigate the specificity of protein complexes on these oligonucleotides, we performed competition experiments with unlabeled self (+S) oligonucleotides and nonspecific (+NS) oligonucleotides. We show that the myocyte protein complex formed on the labeled MEF-1 oligonu-

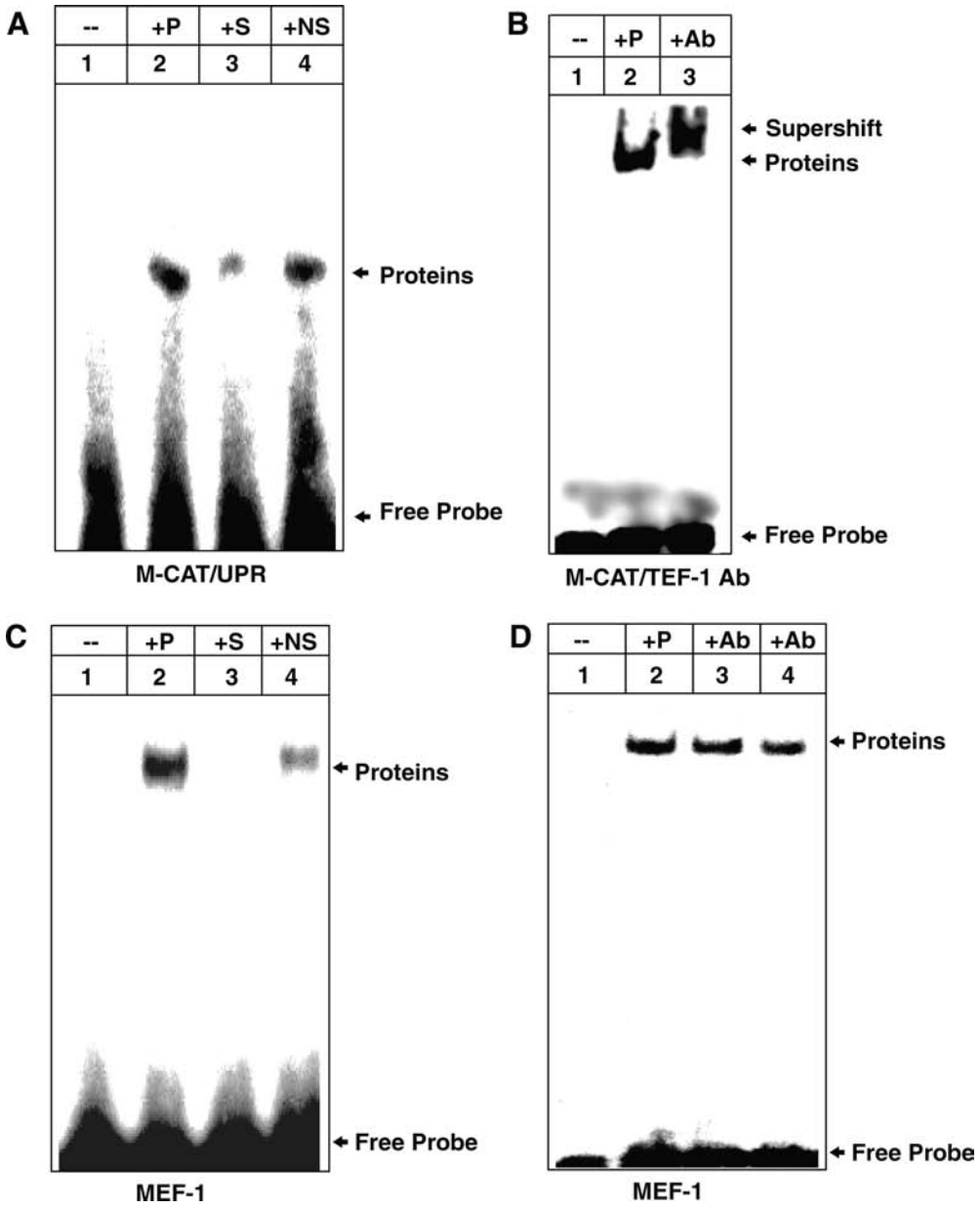

FIG. 2. Upstream promoter region (UPR) electrophoretic mobility shift analysis (EMSA). Myocyte extracts were incubated with the ^{32}P-labeled oligonucleotides from the UPR and analyzed by EMSA. Lane 1 (--) does not contain proteins. Lanes 2, 3, and 4 contain proteins. Lane 3 contains a 100-fold excess of unlabeled self (+S) oligonucleotides. Lane 4 contains a 100-fold excess of nonspecific (+NS) oligonucleotides. (**A**) M-CAT oligonucleotides. (**B**) M-CAT oligonucleotides with TEF-1 antibody. (**C**) MEF-1 oligonucleotides. (**D**) MEF-1 oligonucleotides with MyoD antibodies. Arrows indicate protein complexes bound to labeled oligonucleotides.

cleotide is competed by a 100-fold excess of unlabeled self (+S) oligonucleotides, but is not competed by a 100-fold excess of nonspecific (+NS) oligonucleotide, demonstrating that the protein complex is specific (Fig. 2C). Investigators have shown that MyoD1 proteins bind to the MEF-1 element (Lassar *et al.*, 1989). Therefore, we added MyoD1 antibodies to the protein complex on MEF-1 oligonucleotides (Fig. 2D). We did not observe a supershift with the addition of the MyoD antibody, but instead observed a reduction in cardiac myocyte proteins binding to the MEF-1 oligonucleotide (Fig. 2D).

With removal of 150 bp at the 3′ end of the promoter, γ_3-luciferase gene transcription decreased to control levels. By EMSA, we observed the binding of several myocyte protein complexes to DPR oligonucleotides (data not shown). To measure myocyte protein binding to individual transcription factor binding elements in the DPR, we designed overlapping oligonucleotides, which span identified transcription factor elements in the DPR (Fig. 1C). Using an M-CAT/E oligonucleotide spanning overlapping E-box and two M-CAT elements from the DPR, we observed that proteins form three complexes, designated as A, B, and NS (Fig. 3A, lane 2). The A

and B protein complexes were reduced when competed with unlabeled self (+S), while the bottom (NS) complex was enhanced (Fig. 3B, lane 3). The bottom protein (NS) complex was competed by nonspecific (+NS) oligonucleotides, demonstrating that it is a nonspecific protein complex (Fig. 3A, lane 6). These data demonstrate that the myocyte protein complexes A and B are specific for the overlapping E-box and two M-CAT elements (Fig. 3A). The M-CAT/R (+C/R) oligonucleotides, which contain two M-CAT elements and mutant E-box, were used for competition studies with the labeled M-CAT/E oligonucleotides. Competition with unlabeled + C/R oligonucleotides eliminated the B complex and reduced complex A, but did not affect the nonspecific (NS) complex, demonstrating that the two M-CAT elements are able to compete the myocyte complexes in complexes A and B (Fig. 3A, lane 4). To determine if proteins that specifically recognize only the M-CAT element were present in myocyte A and B complexes, we competed with unlabeled + C/D oligonucleotides that contain an M-CAT element, but do not contain an E-box. Competition with excess + C/D oligonucleotides eliminated complex B and reduced complex A to an extent similar to self M-CAT/E oligonucleotides (Fig. 3A, lane 5). Both M-CAT–containing oligonucleotides (+C/R and + C/D) abolish complex B and reduce complex A on the M-CAT/E oligonucleotides, demonstrating that M-CAT binding proteins are present in the myocyte protein complexes A and B (Fig. 3A, lanes 4 and 5). The + C/D oligonucleotides contain one M-CAT element, but do not contain an E-box element, demonstrating that the M-CAT element is responsible for the competition of complex A and B. These data provide evidence that M-CAT binding proteins are present in myocyte complexes A and B. To further evaluate myocyte protein binding to M-CAT elements within the promoter, we labeled M-CAT/DPR oligonucleotides, which contain an M-CAT element from the DPR, which does not contain an E-box (Berberich *et al*., 1993; Gupta *et al*., 1994). In Figure 3B, we observed one major myocyte complex binding to the M-CAT/DPR oligonucleotides (lane 2). The protein complex on the M-CAT/DPR oligonucleotides was competed by unlabeled self (+S) oligonucleotides, but were unaffected by nonspecific (+NS) oligonucleotides (Fig. 3B, lanes 3 and 4). These studies confirm that proteins bind to M-CAT elements in the promoter (Gupta *et al*., 1994).

In the DPR, there is an exact consensus transcription factor binding element for USF1, which is immediately upstream of the transcription start site (USF1; Xiao *et al*., 2002). In Figure 4A, we observed that one major myocyte protein complex binds to USF oligonucleotides. The protein complex was abolished when competed by self USF oligonucleotides (Fig. 4A, lane 3), but the complex was unaffected by nonspecific (+NS) oligonucleotides, showing that the myocyte complex is specific for its sequence (Fig. 4A, lane 4). In Figure 4B, we explored whether the addition of a USF1 antibody to the EMSA protein complex would cause a supershift or whether it would cause inhibition of the USF proteins binding to the USF oligonucleotide. We observed that addition of the USF1 antibody caused a twofold reduction in the binding of USF proteins to the USF oligonucleotides (Fig. 4B).

Cardiac gene expression

To compare the γ_3 promoter strength with a known cardiac-specific promoter, we transfected the α-MHC-luciferase reporter construct into cardiac myocytes and fibroblasts.

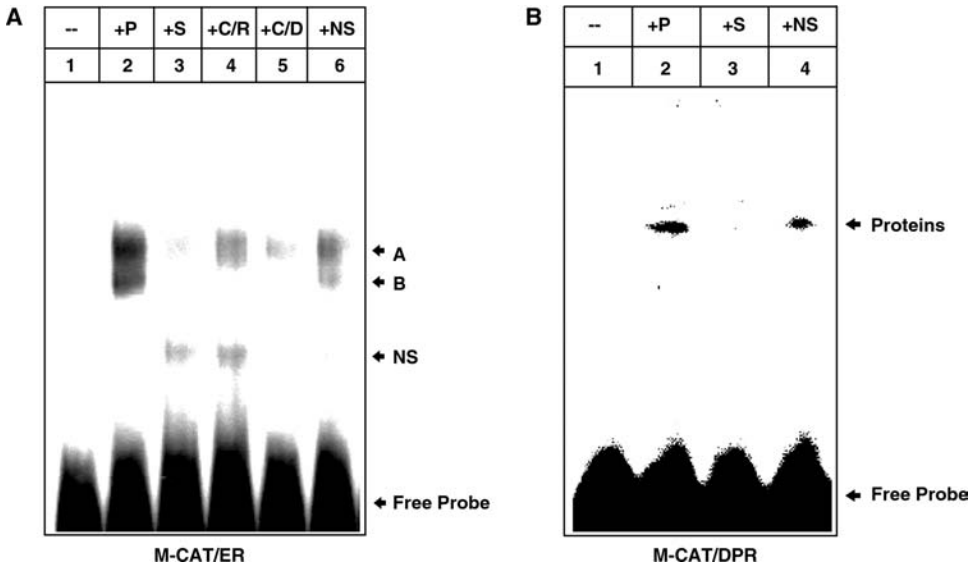

FIG. 3. Downstream promoter region (DPR) electrophoretic mobility shift analysis (EMSA). Myocyte extracts were incubated with the ^{32}P-labeled oligonucleotides from the DPR and analyzed by EMSA. All lanes contain the labeled oligonucleotides. Lane 1 (--) does not contain proteins. Lanes 2, 3, and 4 contain proteins. Lane 3 contains a 100-fold excess of unlabeled self (+S) oligonucleotides. Lane 4B contains a 100-fold excess of nonspecific (+NS) oligonucleotides. Arrows indicate protein complexes bound to labeled oligonucleotides. (**A**) M-CAT/E oligonucleotides contain M-CAT and E-box elements. Lane 4 contains a 100-fold excess of +C/R oligonucleotides. Lane 5 contains a 100-fold excess of +C/D oligonucleotides. Lane 6 contains a 100-fold excess of nonspecific (+NS) oligonucleotides. (**B**) M-CAT/DPR oligonucleotides.

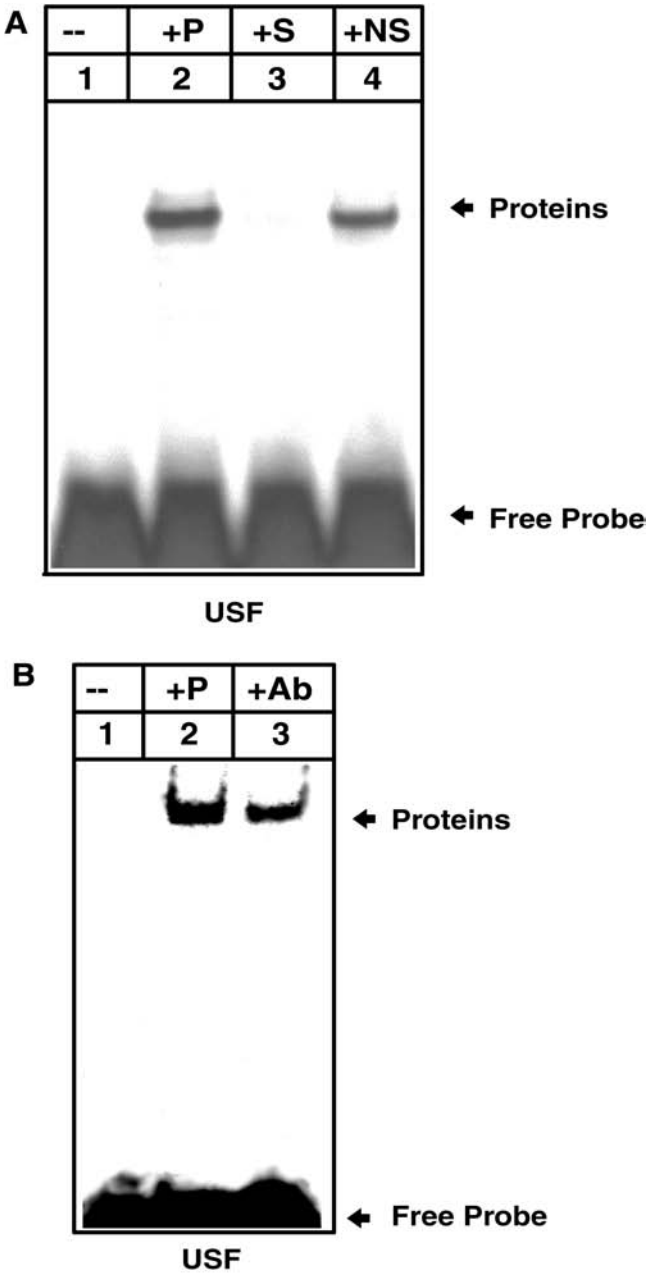

FIG. 4. Upstream stimulatory factor 1 (USF1) electrophoretic mobility shift analysis (EMSA). Myocyte extracts were incubated with the ^{32}P-labeled oligonucleotides from the DPR and analyzed by EMSA. All lanes contain the labeled oligonucleotides. (**A**) USF oligonucleotides. Lane 1 (--) does not contain proteins. Lanes 2, 3, and 4 contain proteins. Lane 3 contains a 100-fold excess of unlabeled self (+S) oligonucleotides. Lane 4 contains a 100-fold excess of nonspecific (+NS) oligonucleotides. (**B**) USF1 antibody. Lane 1 does not contain proteins. Lanes 2 and 3 contain myocyte proteins. Lane 3 contains the addition of USF1 antibodies to the complex. Arrow(s) indicate protein complexes bound to labeled oligonucleotides.

In Figure 5, the α-MHC-promoter-luciferase reporter demonstrated a 5.0-fold ± 0.40 increase in luciferase gene expression compared to control and no expression in fibroblasts, demonstrating cardiac-specific expression. The p(*Sac*I)-γ_3-luciferase reporter construct showed 4.8-fold ± 0.40 in cardiac myocytes and no expression in fibroblasts above control levels, demonstrating that γ_3-luciferase reporter activity is comparable to α-MHC-luciferase expression in cardiac myocytes (Fig. 5). The p(*Eco*RI)-γ_3-luciferase reporter construct also shows enhanced luciferase activity in cardiac myocytes, but no activity in fibroblasts. These data demonstrate that γ_3-luciferase constructs display strong gene expression in cardiac myocytes.

Discussion

We have identified two important regulatory regions in the promoter, which are required for γ_3 expression in cardiac myocytes. The removal of the 48 bp UPR from the 1.0 kb promoter resulted in a 75% reduction in γ_3 luciferase gene expression in cardiac myocytes. The UPR contains two myocyte-specific transcription factor elements, that is, M-CAT and MEF-1. The 150 bp DPR contains the reported transcription start site for the γ_3 gene in mouse and human species (Schwindinger *et al.*, 2004), and its removal abolished all transcription activity, which demonstrates that the DPR contains the core promoter. Using the reported transcription start site (Schwindinger *et al.*, 2004), the DPR contains 88 bp upstream and 43 bp downstream of the transcription start site, which overlaps a consensus INR element (Kraus *et al.*, 1996) (Table 1). The DPR has 60% G + C content, does not contain a TATA-box, but does contain a USF1 element 24 bp upstream of the INR element and the transcription start site (Table 1).

The UPR and DPR contain multiple M-CAT transcription factor elements (Gupta *et al.*, 1994). The M-CAT transcription

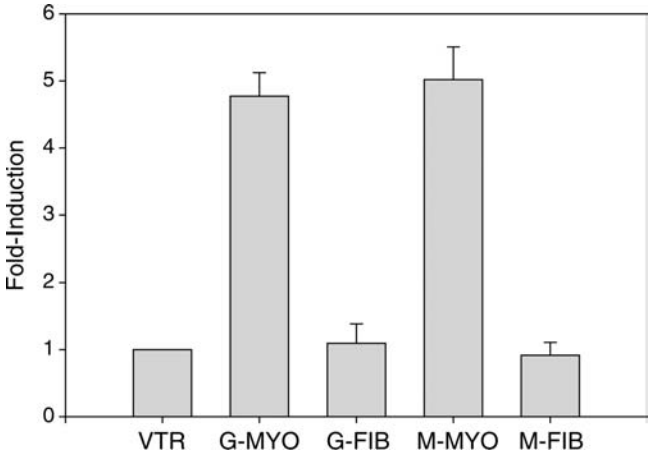

FIG. 5. Comparison of γ_3 promoter and α-MHC promoters. Luciferase gene expression was measured in cardiac myocytes and fibroblasts. The reported fold induction is the relative change in luciferase reporter gene expression between the pGL3-basic vector and the cells transfected with luciferase constructs ($n = 5$). VTR, pGL3-basic (no promoter) vector; G-MYO, γ_3 in cardiac myocytes; G-FIB, γ_3 in fibroblasts; M-MYO, α-MHC in cardiac myocytes; M-FIB, α-MHC in fibroblasts.

factor elements are highly conserved between mouse and human species, suggesting that the myocyte-specific M-CAT elements are important for γ_3 gene expression in cardiac myocytes. Studies have shown that myocyte-specific M-CAT elements are required for the expression of cardiac genes in the heart (Gupta *et al*., 1994; Molkentin and Markham, 1994). Myocyte-specific M-CAT elements have been shown to regulate the cardiac α-MHC promoter (Gupta *et al*., 1994), the cardiac β-myosin heavy chain (β-MHC) promoter (Giger *et al*., 2002), human cardiac phospholamban promoter (McTiernan *et al*., 1999), cardiac B-type natriuretic peptide promoter (Thuerauf and Glembotski, 1997), the cardiac troponin T promoter (Butler and Ordahl, 1999), and the cardiac troponin C promoter (Larkin *et al*., 1996). The cardiac troponin T promoter contains two M-CAT elements in tandem (Butler and Ordahl, 1999). In the UPR, the two M-CAT elements are tandem to one another; in the DPR, two of the M-CAT elements are separated by only 4 bp. Two of the three M-CAT elements in the DPR are identical to the M-CAT elements in the cardiac β-MHC and mouse cardiac Troponin C promoters (Larkin *et al*., 1996). TEF-1 transcription factor binds to M-CAT elements in the promoters of many genes expressed in cardiac muscle cells (Larkin *et al*., 1996; Stewart *et al*., 1998).

The UPR contains a 14 bp myocyte-specific MEF-1 consensus element, which binds cardiac myocyte proteins (Buskin and Hauschka, 1989; Horlick and Benfield, 1989; Amacher *et al*., 1993; Apone and Hauschka, 1995; Feo *et al*., 1995; Klamut *et al*., 1997). The MEF-1 has been shown to positively regulate transcription from muscle and/or cardiac promoters, that is, muscle creatine kinase (Buskin and Hauschka, 1989; Amacher *et al*., 1993), β enolase gene (Feo *et al*., 1995), and muscle/cardiac-specific form of the dystrophin gene (Klamut *et al*., 1997). Investigators have reported that MyoD binds the 14 bp MEF-1 element and that MyoD antibodies recognize MEF-1 proteins (Lassar *et al*., 1989). Thus, the 48 bp UPR, which accounts for 75% of the promoter activity in cardiac myocytes, contains two myocyte-specific transcription factor elements.

In addition to the myocyte-specific transcription factor elements identified, the 1.0 kb promoter contains one USF element that is immediately upstream of the transcription start site and INR element. The USF1 elements have been shown to be important regulatory elements in cardiac promoters (Makaula *et al*., 2006). The USF1 transcription factor has been shown to regulate the cardiac isoform of the acetyl-CoA carboxlyase promoter (Makaula *et al*., 2006), enhance the contractile-mediated activity of the cardiac α-MHC promoter (Xiao *et al*., 2002), and control the expression of the cardiac ventricular myosin light-chain 2 promoter (Navankasattusas *et al*., 1994). It is reported that USF1 proteins can act as a docking proteins to recruit basal transcription factors to facilitate formation of the transcription preinitiation complex (Roy *et al*., 1991), and have been shown to interact with TFIID to stimulate gene transcription (Kokubo *et al*., 1993). The USF element in the DPR is 24 bp upstream of the INR element and the TFIID element (Table 1), suggesting that it may play a similar role in the γ_3 promoter.

Table 1. Consensus Transcription Factor Sequences

USF, histone4 (Xiao *et al*., 2002)	TGACTC
MEF-1 (Buskin and Hauschka, 1989; Horlick and Benfield, 1989)	CNAGCACCTGCCNG
E-box/MybB (Berberich *et al*., 1993)	CAGTTG
M-CAT (Gupta *et al*., 1994)	(G/A)(G/A)(T/C)ATG
TFIIB/D & INR (Kraus *et al*., 1996)	TGCGCCAGAATC

In summary, we have shown that the γ_3 luciferase constructs are expressed at high levels in cardiac myocytes, while showing no activity in fibroblasts. The γ_3 promoter shares myocyte-specific transcription factor elements with the α-MHC promoter. The UPR, which accounts for 75% of the promoter activity in cardiac myocytes, contains myocyte-specific M-CAT and MEF-1 elements. The DPR contains USF1, INR elements, and the transcription start site. We have shown that myocyte proteins specifically bind to MEF-1, M-CAT, and USF elements to regulate γ_3 gene expression in cardiac myocytes.

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