Regulation of the Calreticulin Gene by GATA6 and Evi-1 Transcription Factors[†]

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ABSTRACT: Calreticulin is a Ca^{2+}-buffering chaperone of the endoplasmic reticulum. The protein is highly expressed in embryonic heart but downregulated in postnatal heart, indicating that expression of calreticulin in the heart is highly regulated. In this study we identify GATA6 and Evi-1 transcription factors as new regulators of the calreticulin gene. In neonatal rat ventricular cardiomyocytes and mouse fibroblasts the calreticulin gene is activated by GATA6 but repressed by Evi-1. Furthermore, transactivation of the calreticulin gene by GATA6 is suppressed by Evi-1, suggesting an antagonistic role between both GATA6 and Evi-1. Using EMSA, ChIP analysis, and site-specific mutagenesis, we showed that GATA6 and Evi-1 bind to site 1 on the calreticulin promoter. GATA6 and Evi-1 are highly expressed early during cardiogenesis of ES cells, suggesting that they may regulate expression of the calreticulin gene during cardiac development.

Calreticulin is a Ca^{2+}-binding chaperone in the endoplasmic reticulum (ER)[1] which plays an important role during embryogenesis. Calreticulin deficiency is embryonic lethal due to impaired cardiac development (*1*), and cardiogenesis is inhibited in calreticulin-deficient ES cells (*2*). Calreticulin is significantly downregulated in the postnatal heart (*1*), and overexpression of the protein in newborn hearts results in complete heart block and death (*3*). In ES cells, upregulation of the protein leads to impaired development of the cardiac conductive system (*4*). Taken together, these findings indicate that calreticulin is important for heart development, and its expression is highly regulated during embryogenesis. We have reported that the calreticulin gene is a target for the Nkx2.5 cardiac-specific transcription factor and COUP-TF1 (*5*). COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene in the postnatal heart to suppress expression of the calreticulin gene (*5*). Moreover, the calreticulin gene is also regulated by a cardiac-specific transcription factor myocyte-enhancer factor 2C (MEF2C) during cardiogenesis (*6*). Therefore, expression of the calreticulin gene appears to be regulated by several transcription factors in embryonic heart.

The transcription factor GATA6 is expressed in the developing heart and plays an important role in cardiac development (*7, 8*). However, to date only two genes have been identified as cardiac-specific targets of GATA6: cardiac troponin C (*9*) and Wnt2 (*10*). The calreticulin promoter contains a putative GATA binding site(s), which indicates that the calreticulin gene may be regulated by GATA6. Ecotropic viral integration site 1 (Evi-1) is another transcription factor involved in mouse embryogenesis (*11*). Importantly, Evi-1 binds a consensus nucleotide sequence which is very similar to the GATA binding site (*12, 13*), suggesting that Evi-1 may also be involved in regulation of the calreticulin gene.

In this study, we show that the transcription factor GATA6 activates and the transcription factor Evi-1 represses the calreticulin promoter both in the cardiomyocytes and in fibroblasts. Using ChIP, EMSA, and mutagenesis analyses, we showed that GATA6 and Evi-1 bound to the same site on the calreticulin promoter. This binding site corresponded to the GATA site 1 of the calreticulin promoter. Analysis of the expression of GATA6 and Evi-1 during cardiomyocyte differentiation of ES cells indicated that these genes express at early stages of cardiogenesis and they may play a role in regulation of the calreticulin gene.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis. Plasmid pLC1 containing luciferase reporter gene under control of the calreticulin promoter was generated as described previously (*14*). Site-directed mutation of the double GATA binding site in the calreticulin promoter was performed using the QuickChange site-directed mutagenesis kit from Stratagene. Specifically, the TTATCT nucleotide sequence was mutated to TCTAGA, and the CTATCA nucleotide sequence was mutated to CGCGTA.

Cell Culture and Transfection. Rat primary cardiomyocytes were isolated from neonatal rats as described previously (*6, 15*). Cardiomyocytes and NIH3T3 cells were cultured in Dul-

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[1] Abbreviations: ER, endoplasmic reticulum; ES, embryonic stem; EB, embryoid body; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1; MEF2C, myocyte-enhancer factor 2C; Evi-1, ecotropic viral integration site 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay.

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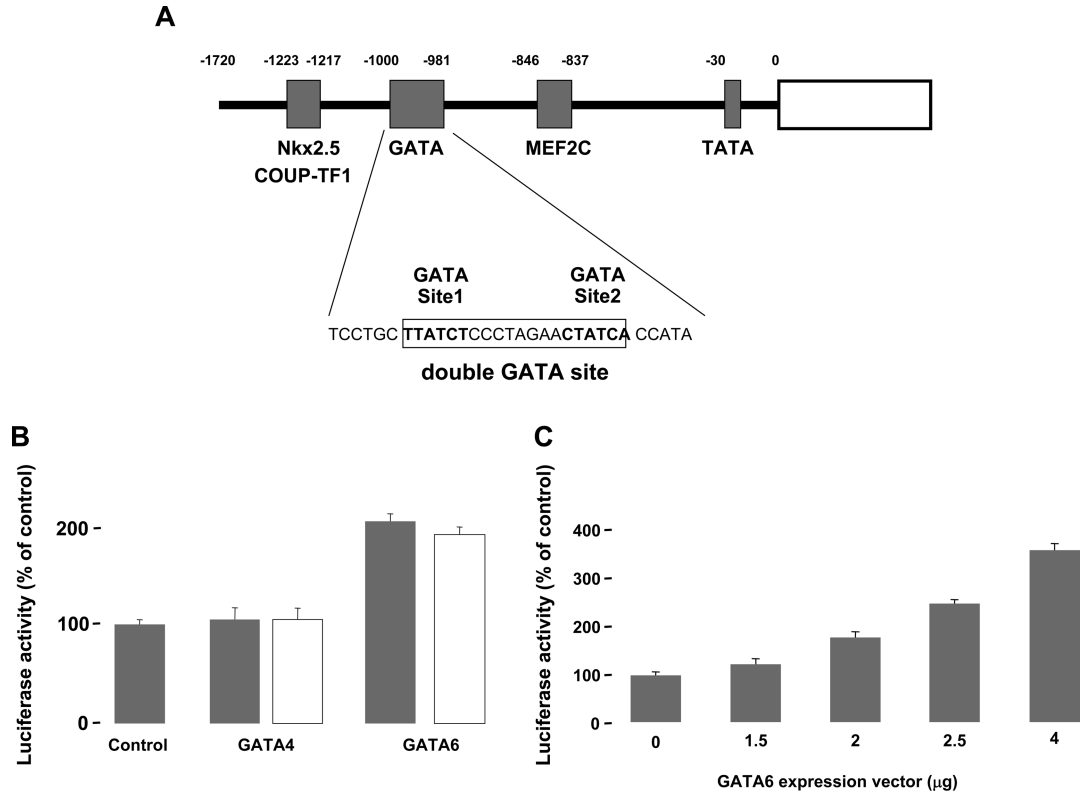

FIGURE 1: Activation of the calreticulin promoter by GATA6. (A) The location of the Nkx2.5 and COUP-TF1 site (*5*), the double GATA site (this work), the MEF2C site (*6*), and the TATA box is indicated. (B) NIH3T3 cells (filled bars) or neonatal rat primary cardiomyocytes (empty bars) were cotransfected with luciferase reporter construct containing 1.8 kb of the calreticulin promoter (pLC1), the β-galactosidase expression vector, and the GATA6 expression vector. (C) NIH3T3 cells were cotransfected with pLC1 vectors, the β-galactosidase expression vector, and increasing concentrations of the GATA6 expression vector DNA. The transfection efficiency for cardiomyocytes and fibroblasts was approximately 30% and 60%, respectively. Luciferase activity was normalized against β-galactosidase. Data are means ± SD of three independent experiments.

becco's modified Eagle's medium supplemented with 10% bovine growth serum at 37 °C with 5% CO_2. After 24 h the cells were transfected at 80% confluency using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. The transfection efficiency for fibroblasts and cardiomyocytes was approximately 60% and 30%, respectively. Mouse ES cells (CGR8) were propagated in multicell DMEM supplemented with sodium pyruvate, nonessential amino acids, mercaptoethanol, 7.5% fetal bovine serum, L-glutamine, and leukemia inhibitory factor (*4*). ES cell differentiation was performed as described previously (*4*).

Yeast One-Hybrid. An 11 day mouse embryo Matchmaker cDNA library (Clontech) was screened for interactions with double GATA binding response elements on the calreticulin promoter (Figure 1A) (*5*). Three tandem repeats of this element (5′-**TTATCT**CCCTAGAA**CTATCA**-3′; GATA site depicted in bold and underlined) were cloned into *Eco*RI and *Xba*I restriction sites of pHISi, and into *Eco*RI and *Sal*I sites of pLacZi, generating pHIS-GATA and pLacZ-GATA, respectively. Screening of the library was carried out as recommended by the manufacturer.

Luciferase and β-Galactosidase Assay. After transfection, cells were grown for 48 h and harvested in NP40 buffer containing 100 mM Tris, pH 7.8, 0.5% NP40, and 0.5 mM DTT. Luciferase and β-galactosidase activity were measured as described previously (*14*). Luciferase activity was normalized with β-galactosidase activity to correct the variations in transfection efficiency.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) assay was performed as described previously (*6*). Briefly, cells were transiently transfected with pcDNA-GATA6-myc-His expression vector or HA-Evi-1 expression vector and cross-linked in 1% formaldehyde at room temperature for 20 min. Cells were then lysed with the Extract-N-Amp kit (Sigma) according to the manufacturer's instruction. Chromatin was sheared by sonication followed by centrifugation for 10 min. Supernatants were precleared with protein−Sepharose A beads for 1 h at 4 °C. Immnunoprecipitation was performed with mouse anti-His or anti-HA antibodies at 4 °C overnight. DNA was purified and analyzed by PCR using the following primers: forward, 5′-CAGGTACTGTTCTTCCTCCTCC-3′, and reverse, 5′-GCTGTCCACGGTTCAAGAGC-3′. PCR reaction was carried out as follows: denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. ChIP analysis of Evi-1 binding to the calreticulin promoter in myeloid cell lines NFS50 and 32Dcl12 was carried out as described previously (*16*). Immunoprecipitation was with rabbit polyclonal antibodies specific for the N- and C-terminal region of Evi-1 (*16*). Primers for PCR amplification were as follows: forward, 5′-GGTTCCCAATTCCTCAGTTTGG-3′, and reverse, 5′-AGGAGTCACTTCTTGTGGTCTC-3′. For all analyses three independent ChIP experiments were carried out.

Electrophoretic Mobility Shift (EMSA) Assay. Full-length GATA6, Evi-1, and luciferase proteins were synthesized using a coupled transcription and translation TNT reticu-

lolysate system. Synthetic oligodeoxynucleotides corresponding to the GATA6 site 1 binding nucleotide sequence (5′-TCCTGC**TTATCT**CCCTAG-3′) and the GATA6 site 2 nucleotide sequence (5′-CTAGAA**CTATCA**CCATAG-3′) were used. The nucleotide sequence corresponding to the GATA6 binding site in the histidine decarboxylase promoter was used as a positive control: 5′-TACTGC**TGATAA**GGAAA-3′ (*17*). The synthetic oligodeoxynucleotide (5′-GTATTTTAGT**GACAAGATA**GTAGAGAATCT-3) corresponding to the Evi-1 binding site of the inositol trisphosphate type 2 receptor promoter was used as a positive control for Evi-1 EMSA analysis (*19*). Oligodeoxynucleotides were labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase. EMSA was carried out as described (*5*).

Cardiogenesis of ES Cells and Reverse Transcription PCR Analysis. Mouse ES cells were differentiated to cardiomyocytes as described previously (*4*). Total RNA was isolated from ES cells and embryoid bodies (EB) at different stages of differentiation using Trizol reagent (Invitrogen) (*4*). Total RNA (200 ng) was used for RT-PCR. The PCR amplification was carried out as follows: denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The following primers were used for different genes: GATA6, forward, 5′-GCCAACTGTCACACCACAAC-3′, and reverse, 5′-GTAGAGGCCGTCTTGACCTG-3′; Evi-1, forward, 5′-CTTTGAATCCAAGGCAGAGC-3′, and reverse, 5′-TAGGGTCCGTGAAAACCTTG-3′; α-MHC, forward, 5′-GGAAGAGCGAGCGGCGCATCAAGG-3′, and reverse, 5′-CTGCTGGAGAGGTTATTCCTCG-3′; MEF2C, forward, 5′-AGATACCCACAACACACCACGCGCC-3′, and reverse, 5′-ATCCTTCAGAGAGTCGCATGCGCTT-3′; β-tubulin, forward, 5′-CCGGACAGTGTGGCAACCAGATCGG-3′, and reverse, 5′-TGGCCAAAAGGACCTGAGCGAACGG-3′.

RESULTS

GATA6 Activates the Calreticulin Promoter in Mouse Fibroblasts and Rat Primary Cardiomyocytes. Figure 1A shows that the calreticulin promoter contains two GATA binding sites, suggesting that GATA transcription factors may regulate the gene. To test this, rat primary cardiomyocytes and NIH3T3 fibroblasts were cotransfected with the GATA6 expression vector and a luciferase reporter gene vector (pLC1), which is controlled by 1.8 kilobases (kb) of the calreticulin promoter. pSVβ-galactosidase was used as an internal control. Panels B and C of Figure 1 show that GATA6 significantly induced the luciferase activity in both rat primary cardiomyocytes and NIH3T3 cells whereas GATA4 did not. Cells transfected with promoterless control plasmids showed no detectable luciferase activity (data not shown). Additional EMSA and ChIP analysis revealed that GATA4 did not bind to the GATA sites in the calreticulin promoter (data not shown). Activation of the calreticulin promoter by GATA6 was dose-dependent up to 3.5-fold (Figure 1C). These findings indicate that GATA6 activates transcription of the calreticulin gene.

We have previously reported that cardiac-specific transcription factor NKx2.5 activates the calreticulin gene in the heart while COUP-TF1 acts as a repressor of this gene (*5*).

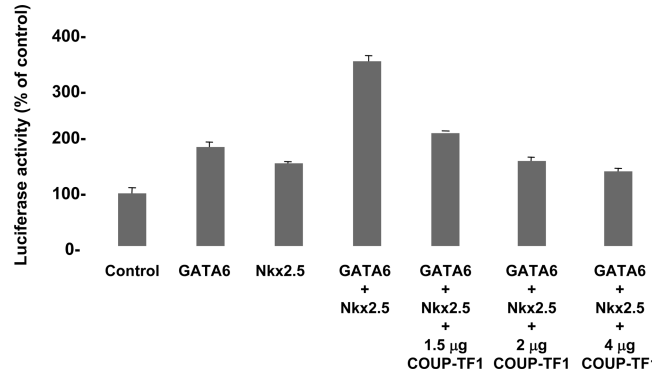

FIGURE 2: Regulation of the calreticulin promoter by GATA6, Nkx2.5, and COUP-TF1. NIH3T3 cells were cotransfected with a luciferase reporter gene controlled by the calreticulin promoter (pLC1) with the GATA6 expression vector, the Nkx2.5 expression vector, and indicated amounts of COUP-TF1 expression vectors. Luciferase activity was normalized against β-galactosidase. Data are means ± SD of three independent experiments.

GATA transcription factors also play a key role in cardiac development (*7, 20*), and most importantly, they affect function of other cardiac transcription factors including Nkx2.5 and MEF (*20–23*). Figure 2 shows that Nkx2.5 synergizes GATA6-mediated activation of the calreticulin gene and COUP-TF1 represses this activation. Thus, it appears that both Nkx2.5 and GATA6 can regulate expression of the calreticulin gene in the heart and they may activate the calreticulin gene individually at different stages or they play synergistic roles at specific stages during cardiac development.

GATA6 and Evi-1 Interact with the Calreticulin Promoter. To demonstrate that GATA6 binds to the double GATA binding site in the calreticulin promoter, we carried out the yeast one-hybrid screen. A total of 1.4×10^6 yeast transformants were screened, and eight positive clones were isolated, which grew on His$^-$/Leu$^-$ plus 15 mM 3-amino-1,2,4-triazole for 7 days at 30 °C. Two of the eight positive clones encoded the GATA6 gene, supporting our observation that GATA6 bound to the calreticulin promoter. Most importantly, three of the eight positive clones encoded Evi-1, a zinc finger transcription factor implicated in leukemic transformation of hematopoietic cells (*24*), indicating that Evi-1 also binds to the double GATA site in the calreticulin promoter. This suggested that calreticulin may be a target for the Evi-1 transcription factor.

To test if Evi-1 regulates the calreticulin promoter, NIH3T3 fibroblasts were cotransfected with the Evi-1 expression vector and a luciferase reporter gene vector (pLC1), which is controlled by the calreticulin promoter. pSVβ-galactosidase was used as an internal control. Figure 3A shows that Evi-1 reduced luciferase activity by 60%, indicating that this transcription factor repressed the calreticulin promoter. Increased level of expression of Evi-1 did not have any additional effect on repression of the calreticulin promoter (Figure 3B). Since Evi-1 bound to the double GATA site on the calreticulin promoter, we tested if Evi-1 affects GATA6-dependent activation of the promoter. NIH3T3 cells were cotransfected with expression vectors encoding Evi-1 and GATA6 followed by measurement of luciferase activity. Expression of GATA6 and Evi-1 resulted in 34% inhibition of GATA6-dependent activation of the calreticulin promoter (Figure 3A). Thus, these results suggest that

GATA6 is a transcriptional activator for the calreticulin gene while Evi-1 is a transcriptional repressor. GATA6 and Evi-1 may play antagonistic roles in regulation of the calreticulin gene.

GATA6 and Evi-1 Bind to the Calreticulin Promoter in Vivo. To examine the interaction between calreticulin promoter and GATA6/Evi-1 *in vivo*, we performed ChIP assay. NIH3T3 cells were transfected with the expression vector encoding His-tagged GATA6 (Figure 3C) or HA-tagged Evi-1 (Figure 3D); transcription factor−DNA complexes were then immunoprecipitated with anti-His tag or anti-HA antibodies. Figure 3C (lane 3) shows that the immunoprecipitate obtained from GATA6 expressing cells contained a DNA fragment corresponding to the GATA6 binding site of the calreticulin promoter. The immunoprecipitate from cells expressing Evi-1 also contained a DNA fragment corresponding to the GATA double site of the calreticulin promoter (Figure 3D, lane 3). To further test if Evi-1 binds to the calreticulin promoter, we performed additional ChIP analysis using myeloid 32Dcl3 and NFS58 cells expressing a high level of Evi-1 (Figure 3E). This was tested with two independent anti-Evi-1 antibodies (Figure 3E). Together, these data indicate that GATA6 and Evi-1 bind to the calreticulin promoter in living cells and identify the GATA6 and Evi-1 binding site in the calreticulin promoter at −981 to −1000 (Figure 1A).

GATA6 and Evi-1 Bind to GATA Site 1 of the Calreticulin Promoter. In order to identify the exact site GATA6 and Evi-1 bind to within the double GATA binding nucleotide sequence, we carried out an EMSA. We used two ^{32}P-labeled synthetic oligodeoxynucleotides corresponding to GATA site 1 and GATA site 2 in the calreticulin promoter. The synthetic oligodeoxynucleotide corresponding to the GATA6 binding site of the histidine decarboxylase was used as a positive control (Figure 3F, lane 1). The Evi-1 binding site of the inositol trisphosphate type 2 receptor promoter was used as an Evi-1 positive control (Figure 3F, lane 6). Figure 3F shows that both GATA6 (lane 2) and Evi-1 (lane 7) bound to the GATA site 1 of the calreticulin promoter. The binding was significantly reduced in the presence of a 30-fold excess of unlabeled oligodeoxynucleotides (Figure 3F, lanes 3 and 8). neither GATA6 nor Evi-1 bound significantly to GATA site 2 (Figure 3F, lanes 4 and 9). GATA6 bound to the GATA binding element of the histidine decarboxylase promoter (Figure 3F, lane 1) while luciferase did not bind to any of the tested DNA probes (Figure 3F, lanes 13 and 14). We concluded that GATA6 and Evi-1 bind to GATA site 1 of the double GATA binding site of the calreticulin promoter.

To further demonstrate specific binding of GATA6 and Evi-1 to GATA site 1 of the calreticulin promoter, we carried out mutational analysis of the pLC1 vector coupled with luciferase reported gene assay. Figure 4A shows that GATA6 did not activate and Evi-1 did not suppress the calreticulin promoter when GATA site 1 was mutated. In contrast, mutation of GATA site 2 did not influence GATA6-dependent activation of the promoter or Evi-1-dependent suppression (Figure 4B). These observations further support our conclusion that GATA6 and Evi-1 bind to GATA site 1 of the calreticulin promoter.

GATA6 and Evi-1 Are Expressed Early in ES Cell Cardiomyocyte Differentiation. GATA6 is a potential regulator of cardiac development (*25*). Evi-1 has also been reported to be highly expressed in E12.5 mouse heart (*11*). Using RT-PCR techniques, we examined expression of GATA6 and Evi-1 mRNA during ES cell cardiogenesis (*2, 4*). Cardiogenesis of ES cells was completed at day 12 of differentiation as indicated by the presence of over 80% of beating EBs. Total RNA was isolated from day 0 to day 12 during differentiation followed by RT-PCR analysis. Cardiac-specific marker genes, MEF2C and α-MHC, were expressed as early as day 6–8 (Figure 5). GATA6 was already expressed at day 6, an early stage of cardiogenesis, whereas the Evi-1 gene was activated at day 8, a relatively late stage of ES cell cardiogenesis (Figure 5).

DISCUSSION

In this study we identified that the calreticulin gene is a direct target of GATA6 and Evi-1 transcription factors. The calreticulin promoter contains two GATA binding sites (site 1 and site 2). Using EMSA, ChIP analysis, and site-specific mutagenesis, we show that GATA6 and Evi-1 bind to the GATA site 1 on the calreticulin promoter. Importantly, transactivation of the calreticulin gene by GATA6 is suppressed by Evi-1, suggesting an antagonistic role between GATA6 and Evi-1. GATA6 and Evi-1 are expressed early during cardiomyocyte differentiation of ES cells, suggesting they may regulate expression of the calreticulin gene during heart development.

Several genes have been identified as targets of GATA6, such as the Wnt7b gene (*26*) and the surfactant protein A gene (*27*) in lung tissue, the smooth muscle myosin heavy-chain gene (Sm-MHC) in vascular smooth muscle (*28*), and the Wnt2 gene in cardiomyocyte (*10*). These observations suggest that GATA6 may play different transcriptional functions in various tissues. It is well established that GATA6 is important during cardiogenesis (*25, 29*). Overexpression of GATA6 in embryos prevents the differentiation of heart precursors (*29*). Importantly, GATA6 knockout in *Xenopus* and zebrafish embryos blocks cardiac development (*25*). Calreticulin is also essential for cardiogenesis in mice (*1*) and ES cells (*2*). Here we found that GATA6 activates the calreticulin gene in neonatal rat cardiomocytes and in mouse fibroblasts, indicating that the calreticulin gene may be regulated by GATA6 during cardiogenesis. Similar to calreticulin, GATA6 is highly expressed at the early stages of embryonal carcinoma cell cardiac differentiation (*1, 10*), further supporting the conclusion that GATA6 may contribute to upregulation of the calreticulin gene during cardiogenesis. GATA4 and GATA5 transcription factors may also play a role during cardiogenesis or cardiac hypertrophy (*30, 31*). However, GATA4 does not bind to the calreticulin promoter, and it does not activate the calreticulin gene in fibroblasts and cardiomyocytes. GATA5 is expressed in developing heart, yet interestingly mice deficient in GATA4 develop female genitourinary tract abnormalities (*32*).

GATA factors bind to a canonical DNA motif, 5′-(A/T)GATA(A/G)-3′ (*33*). The calreticulin promoter contains two putative GATA sites (Figure 1A). Both sites (5′-

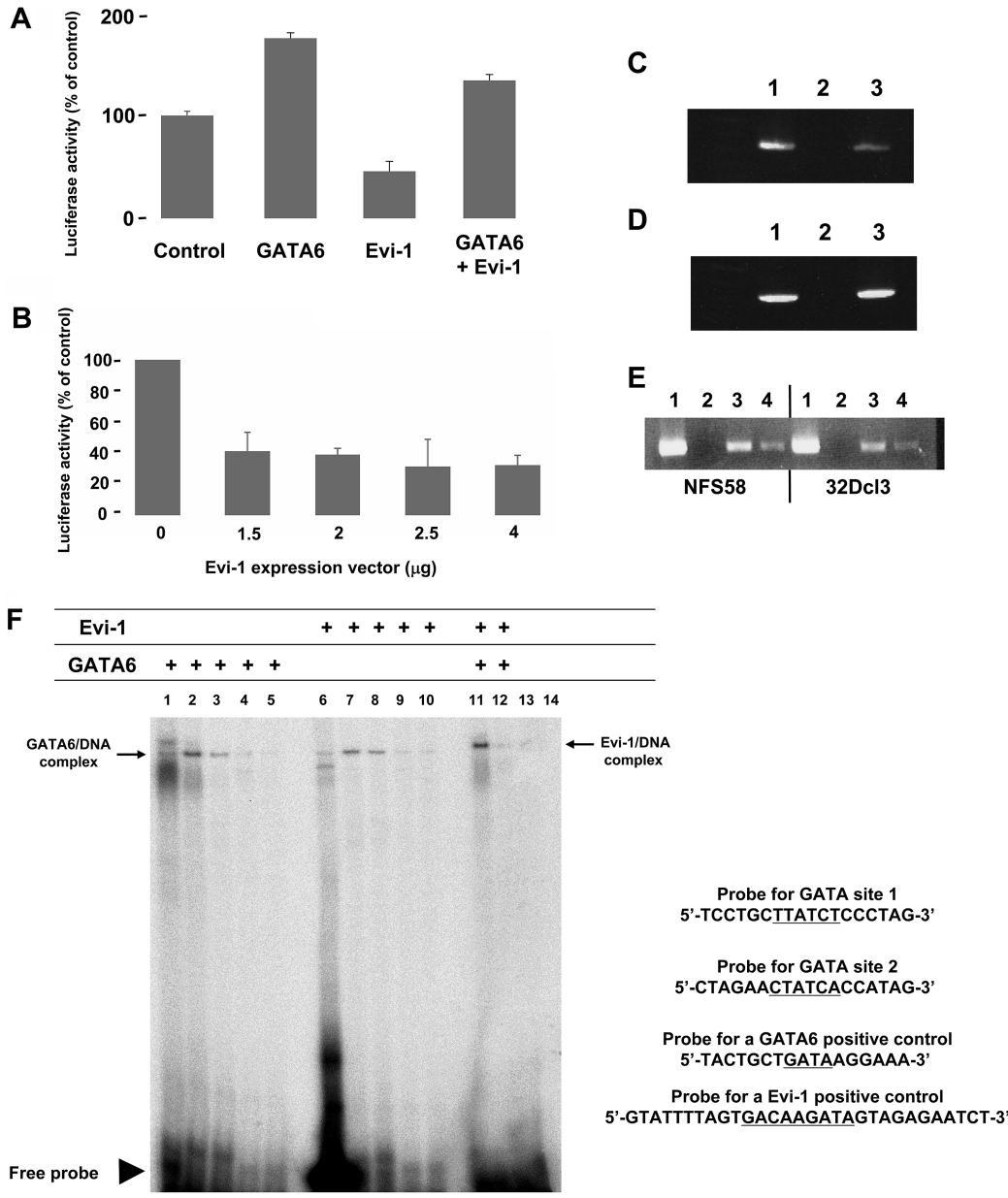

FIGURE 3: GATA6 activates and Evi-1 represses the calreticulin promoter. (A) NIH3T3 cells were transiently cotransfected with the expression plasmids indicated and with a luciferase reporter gene controlled by the calreticulin promoter (pLC1). Luciferase activity was normalized against β-galactosidase. Data are means ± SD of three independent experiments. (B) NIH3T3 cells were cotransfected with pLC1 vectors, the β-galactosidase expression vector, and increasing concentrations of the Evi-1 expression vector DNA. Luciferase activity was normalized against β-galactosidase. Data are means ± SD of three independent experiments. (C, D) ChIP analysis of a putative GATA6 (C) and Evi-1 (D) binding site in the mouse calreticulin promoter. Lanes: 1, input DNA; 2, negative control without primary antibody; 3, with anti-His antibody (C) or anti-HA antibody (D). (E) ChIP analysis of NFS58 and 32Dcl3 cells using either control rabbit serum (lane 2), anti-Evi-1 N-terminus antibodies, or anti-Evi-1 C-terminus antibodies (lanes 3 and 4, respectively) (*16*). Lane 1: input DNA. (F) GATA6 and Evi-1 bind to the GATA site 1 in the calreticulin promoter. EMSA was performed with GATA6, Evi-1, and luciferase (control) that were translated *in vitro*. Synthetic calreticulin promoter GATA site 1 and GATA site 2 elements were used as probes for binding activity. A probe containing the GATA6 binding site of the histidine decarboxylase promoter and the Evi-1 binding site of the inositol trisphosphate type 2 receptor promoter was used as a positive control (lanes 1and 6, respectively). Lanes: 1, binding of GATA6 to positive control probe; 2, binding of GATA6 to GATA site 1 of the calreticulin promoter; 3, GATA6 protein + radiolabeled probe of GATA site 1 with a 30-fold excess of unlabeled probe; 4, binding of GATA6 to GATA site 2 of the calreticulin promoter; 5, GATA6 protein + radiolabeled probe of GATA site 2 with a 30-fold excess of unlabeled probe; 6, binding of Evi-1 to a positive control probe; 7, binding of Evi-1 to GATA site 1 of the calreticulin promoter; 8, Evi-1 protein + radiolabeled probe of GATA site 1 with a 30-fold excess of unlabeled probe; 9, binding of Evi-1 to GATA site 2 of the calreticulin promoter; 10, Evi-1 protein + radiolabeled probe of GATA site 2 with a 30-fold excess of unlabeled probe; 11, binding of Evi-1 and GATA6 to GATA site 1 of the calreticulin promoter; 12, binding of Evi-1 and GATA6 to GATA site 2 of the calreticulin promoter; 13, binding of luciferase control to GATA site 1; 14, binding of luciferase to GATA site 2. The positions of the GATA6−DNA complex and the Evi-1−DNA complex are indicated by the arrows. Nucleotide sequences of synthetic oligodeoxynucleotides used for EMSA analysis are indicated.

TTATCT-3′ and 5′-CTATCA-3′) match perfectly the consensus nucleotide sequence for GATA binding. Our results indicate that only the first GATA site (site 1) in the calreticulin promoter binds GATA6, resulting in activation of the gene. It is not clear at present why site 2 is silent as far as GATA6 transcription factor is concerned. It may be

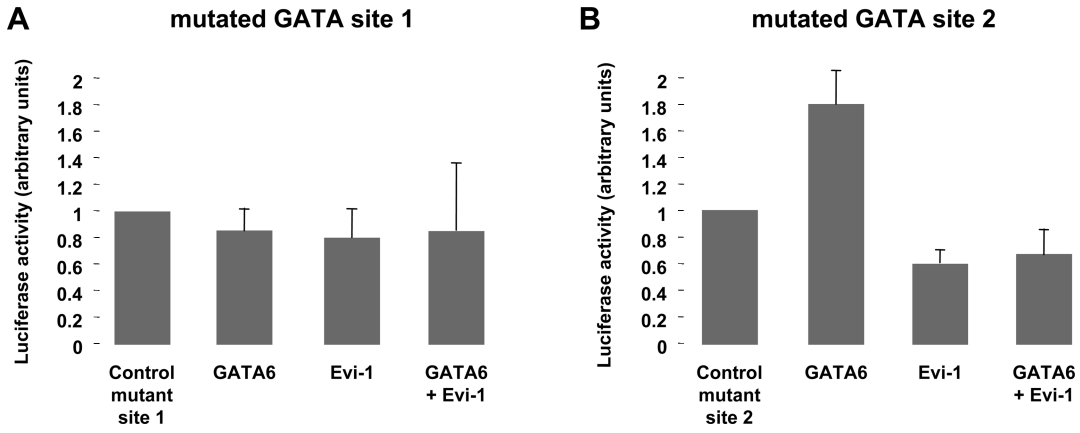

FIGURE 4: Mutational analysis of GATA6 and Evi-1 binding to the calreticulin promoter. Putative GATA6 and Evi-1 binding sites (5′-**TTATCT**CCCTAGAA**CTATCA**-3′) of the calreticulin promoter (plasmid pLC1) were mutagenized to 5′-**TCTAGA**CCCTAGAACTATCA-3′ (mutant GATA site 1) or to 5′-TTATCTCCCTAGAA**CGCGTA**-3′ (mutant GATA site 2). NIH3T3 cells were transiently cotransfected with the expression plasmids indicated and with a luciferase reporter gene controlled by the calreticulin promoter (pLC1) or by the calreticulin promoter with mutated GATA site 1 or GATA site 2. Luciferase activity was normalized against β-galactosidase. Data are means ± SD of three independent experiments.

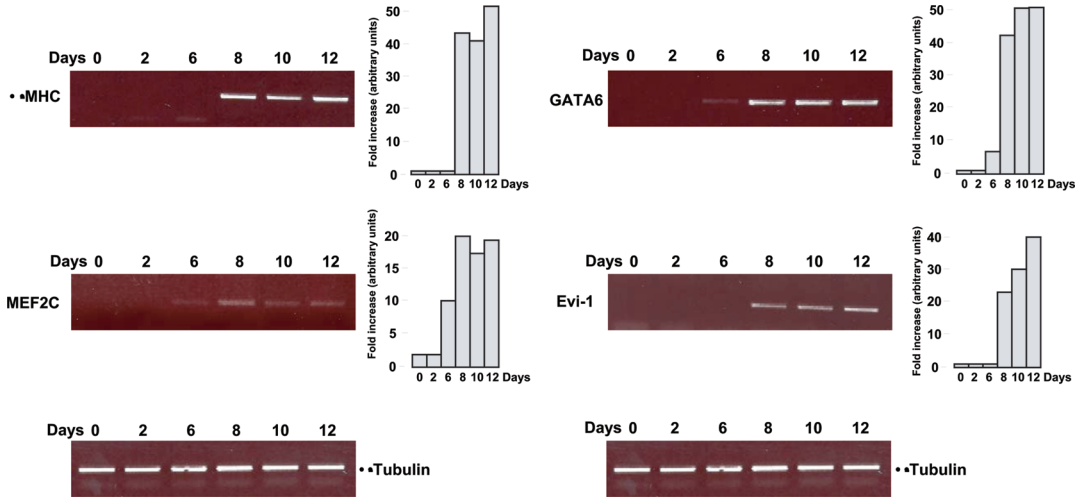

FIGURE 5: Expression of Evi-1, GATA6, and cardiac marker genes during ES cell cardiogenesis. Cardiogenesis of ES cells was carried out as described previously (*4*). Total RNA was isolated from cells at the day of differentiation indicated in the figure followed by RT-PCR. Levels of mRNA were quantified using Image J software. Data were normalized using β-tubulin mRNA.

activated by a different GATA transcription factor in a tissue-specific manner. Importantly, these observations indicate that not all consensus GATA binding DNA motifs are always functional.

One important finding of this study is that the Evi-1 transcription factor binds to GATA site 1 in the calreticulin promoter and suppresses its transcriptional activity. Evi-1 contains two zinc finger domains called ZF1 and ZF2 (*34*). ZF1 recognizes the nucleotide sequence 5′-GA(C/T)AA-GA(T/C)AAGATAA-3′, and ZF2 recognizes the nucleotide sequence 5′-GAAGATGAG-3′ (*12, 13*). Evi-1 performs optimal repressor activity through the ZF1 binding site but less efficiently through the ZF2 binding site (*35*). The ZF1 binding nucleotide sequence is very similar to the GATA transcription binding motif, indicating that Evi-1 may bind to the GATA site. Indeed, Evi-1 binds to the GATA-1 target site and blocks erythopoiesis (*36*). Evaluation of potential target genes for Evi-1 also revealed that it binds to nucleotide sequences containing repeats of the GATA motif (*37*). In this study, we showed that Evi-1 suppresses GATA6-dependent transactivation of the calreticulin gene and that this is mediated by binding to the GATA site (site 1) of the calreticulin promoter. Although both GATA6 and Evi-1 bind to GATA site 1 of the calreticulin promoter, the two transcription factors do not form protein complexes as tested by immunoprecipitation experiments (data not shown).

A role of Evi-1 in transcriptional regulation is not well understood, and only a few Evi-1 target genes have been identified. Evi-1 may act as a repressor or activator of specific genes. The fusion protein AML1-MDS1-EVI1 (AME) produced in acute myeloid leukemia activates the calreticulin gene (*38*), suggesting that in leukemogensis Evi-1 may affect calreticulin expression. Interestingly, this activation of the calreticulin gene is only mediated by the fusion protein AME in cells or patients but not by Evi-1 protein itself. Other reports indicate that Evi-1 functions as a transcriptional repressor. For example, Evi-1 represses TGFβ signaling through interacting with protein Smad3, thereby suppressing transactivation of the target genes by Smad3 (*39*). AML1/EVI1 fusion protein also suppresses transactivation by AML1 (*40*). Evi-1 may have dual transcriptional regulatory properties, as a repressor or as a transactivator. These contrasting functions of Evi-1 may depend on a specific target gene, specific tissue and developmental stage, and specific

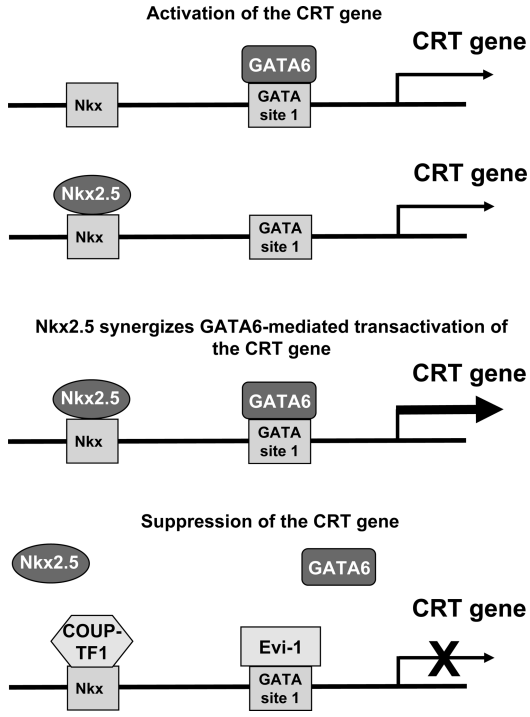

FIGURE 6: A putative model of transcriptional regulation of the calreticulin gene. The figure shows the transcription factors GATA6 and Evi-1 (this study), Nkx2.5, and COUP-TF1 (*5*) play a role in regulation of the calreticulin gene. GATA6 and Evi-1 bind to the GATA site 1 element in the calreticulin promoter. Nkx.2.5 and COUP-TF1 bind to the CRT site 2 element (*5*). In embryonic hearts or ES cell-derived cardiomyocytes GATA6 and Nkx2.5 expression is high, and they activate the calreticulin promoter. Nkx2.5 can synergize GATA6 to activate the transcription of the calreticulin gene. As cardiac development progresses and in newborn hearts Evi-1 and COUP-TF1 bind to GATA site 1 and calreticulin site 2 (*5*), respectively, contributing to repression of the calreticulin gene. CRT = calreticulin.

pathological or physiological conditions. As far as the calreticulin gene is concerned, Evi-1 functions as an effective suppressor of the gene in cultured cardiomyocytes and fibroblasts. By competing with GATA6 for GATA site 1 in the calreticulin promoter, Evi-1 may negatively regulate the calreticulin gene expression during cardiogenesis. The expression pattern of Evi-1 and calreticulin in embryonic and adult tissues indicates an important role for both in mouse development and cardiogenesis (*1, 11*).

We propose that both GATA6 and Evi-1 may play important roles in regulating the calreticulin gene during cardiogenesis (Figure 6) by binding to GATA site 1. As shown in this study, binding of GATA6 to the calreticulin promoter results in activation of the promoter whereas Evi-1 binds to GATA site 1, resulting in suppression of its activity (Figure 6). We previously identified Nkx2.5 (*5*) and MEF2C (*6*) as activators and COUP-TF-1 (*5*) as repressor of the calreticulin gene during cardiac development. Elevated expression of Nkx2.5, MEF2C, and GATA6 during very early cardiac development triggers high expression of calreticulin (Figure 6). COUP-TF-1 antagonizes Nkx2.5-dependent activation of the calreticulin promoter (*5*). In the present study we revealed that Evi-1 suppresses GATA6-dependent activation of the calreticulin gene, indicating that Evi-1 may contribute to the decline of calreticulin levels seen in newborn/mature heart compared with those seen during early stages of cardiogenesis.

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