Regulation of the rat cardiac troponin I gene by the transcription factor GATA-4

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INTRODUCTION

Troponin I (TnI) is a regulatory thin-filament protein which is expressed in striated muscle. Along with the other subunits of troponin and tropomyosin, it participates in the control of muscle activation by Ca$^{2+}$ [1,2]. There are three known TnI genes that encode isoforms that are specific to muscle twitch type in mature animals [3–5]. Cardiac TnI isoform is restricted in its expression to atrial and ventricular cardiac muscle [4]. There is no evidence to suggest that the cardiac isoform is expressed in skeletal muscle at any developmental stage [6]. In contrast, the slow skeletal-muscle TnI gene is expressed in embryonic, fetal and neonatal cardiac muscle, as has been demonstrated in several species [4,7–9]. As development proceeds during gestation, the relative content of cardiac TnI mRNA and protein increases severalfold in both atrial and ventricular tissues, as the slow skeletal-muscle TnI content decreases [4,10,11]. The developmental switch is not complete until 21–28 days postnatally in the rat [4]. Interestingly, the developmental gene switch of the two isoforms in heart is observed and qualitatively similar in both rodents and large mammals including the human [8]. This suggests that the mechanisms that regulate this gene may be common in the rodent and human heart.

In order to characterize the regulation of this cardiac-restricted and developmentally regulated gene, we have characterized the organization and regulatory regions of the rat cardiac TnI gene. The proximal promoter region had the strong ability to direct reporter gene expression in cardiac cells in vitro. These results are in contrast with the skeletal-muscle TnI genes, in which intronic regulatory elements are important in the regulation of reporter gene activity both in vitro and in vivo [12–15]. In addition, multiple DNA-binding sites for the GATA-4 transcription factor were found, and GATA-4 was noted to trans-activate this gene in vivo. An intact proximal GATA site was necessary for transcriptional activation of the gene in vitro.

MATERIALS AND METHODS

Genomic clones

Overlapping genomic clones were obtained from a rat genomic library in Lambda Dash purchased from Stratagene and from a P1 rat genomic library. The phage library was screened by filter hybridization of a random-primer-labelled [16] probe consisting of the rat cardiac cDNA [4]. A single clone containing a 5' portion of the gene was obtained. Subsequently, three clones were obtained by PCR screening of a P1 library [17] using primers from –77 to –58 and antisense to +112 to +133. This screening was performed by Genomic Systems (Manchester, MO, U.S.A.). To facilitate DNA sequencing, fragments digested with restriction enzymes or generated by PCR using the P1 clones as template were placed into pGEM-3Z or pGEM-4Z (Promega) or into TA cloning vector (Invitrogen). Dideoxy DNA sequencing [18] was performed by using Sequenase (US

Abbreviations used: BNP, B naturetic peptide; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility-shift assays; HepG2, Hepatoma G2; MHC, myosin heavy chain; MLC-2, myosin light chain 2; TnI, troponin I; TnC, troponin C; TnT, troponin T.

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The nucleotide sequence in Figure 1 has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U77354.
Biochemical) or by automated sequencing in the Johns Hopkins University Genetics Core facility. The cap site of the gene had been previously characterized by primer extension analysis [4].

Plasmids

Chimaeric plasmids for use in transfection experiments were prepared using upstream regions of the rat cardiac TnI gene cloned into the promoterless vector pGL Basic (Promega) which contains a luciferase reporter gene. Overlapping genomic subclones from the first genomic isolate were first prepared by cloning a 1.2 kb HindIII–EcoRI fragment and a 3 kb EcoRI fragment into pGEM-3Z. The plasmid −150TnI kp GL (−150 to +51) was constructed by digesting the 1.2 kb genomic subclone with ApaI (−150) and BstEII (+51), blunting with T4 polynucleotase, subcloning this into digested blunt pGEM-3Z vector, and subsequently placing the insert directionally into pGL Basic digested with SmaI and SacI. The construct −524TnI kp GL (−524 to +51) was prepared by digesting a 1.2 kb HindIII–EcoRI genomic subclone with BstEII, blunting and subcloning the fragment into XhoI-digested blunt pGL Basic. The orientation was confirmed by sequencing. Because of lack of unique convenient restriction sites, the subsequent subclones were made after using PCR to amplify a 577 bp fragment using the primers corresponding to −532 to −515 and antisense to +16 to +45, but containing an EcoRI site at the 3′ end of the sequence. This fragment was subcloned into pCR®TM (Invitrogen) and subjected to sequencing. The fragment was then digested from the vector with BstEII and EcoRI generating a 565 bp fragment, and subcloned into plasmid 1.2 kb HindIII–EcoRI (genomic subclone) digested with BstEII and EcoRI. The plasmid −896TnI kp GL (−896 to +45) clone was prepared by digesting this chimaeric 1.2 kb clone with HindIII and EcoRI, blunting and cloning into XhoI-digested blunt pGL Basic. Orientation was confirmed by sequencing. The plasmid −2000TnI kp GL was prepared by digesting −896TnI kp GL with KspI (−859) and SacI (in the 3′ linker of pGL Basic) and ligating an upstream 1 kb SacI–KspI fragment from the 3′ EcoRI genomic subclone. Additional plasmids were purchased for use including pGL Control (Promega), which served as a positive control, and cytomegalovirus–β-galactosidase (CMV–β-gal) (Clontech), which served as a control for transfection efficiency. The GATA-4 expression vector pMT+GATA-4 was generously provided by Dr. David Wilson (Washington University, St. Louis, MO, U.S.A.) [19]. Site-directed mutagenesis of the TnI–luciferase clones was performed by the method of Deng and Nickoloff [20] using the Transfornertm kit (Clontech) and confirmed by restriction analysis and sequencing.

Cell culture

Rat cardiomyocyte cultures were prepared using a slight modification of the method of Engelmann et al. [21]. Hearts were removed under sterile conditions from 3-day-old neonatal rats, gently blotted and rinsed in PBS, and then finely minced. Stepwise digestion with collagenase (Wako) was performed by gently rotating the cells in a siliconized glass tube on a wheel in a 37 °C incubator. The supernatant was intermittently removed and spun to collect cells. The first two supernatant fractions were discarded as these contained predominantly non-cardiocytes. The cardiocyte fractions were pooled, filtered through nylon mesh, and then resuspended in the culture medium PC1 (Ventrex). The cells were plated at a density of 2 × 10^6/60 mm plate. Over 90% of the cells were beating within 36 h and stained positively with antibody to cardiac TnI. Hepatoma G2 (HepG2), C2C12, NIH 3T3 and COS 7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum.

Transfections

Cardiocytes were transfected by the use of the liposomal transfection reagent DOTAP (Boehringer-Mannheim), which resulted in higher transfection efficiency than the calcium phosphate method in these primary cells. On the day of isolation of the cardiocytes, 20 μg of test plasmid and 5 μg of CMV–β-gal were added to 50 μl of DOTAP per plate and incubated at room temperature for 10 min. All steps were carried out in duplicate. The mixture was added in a dropwise fashion to the cells and the cells were incubated for 18 h. The medium was then changed and the cells were harvested for assay 72 h after the initial incubation.

HepG2, NIH 3T3, COS 7 and C2C12 cells were transfected by calcium phosphate co-precipitation [22]. In the case of the HepG2 and NIH 3T3 cells, the cells were plated at a density of 2 × 10^6/60 mm plate for transfection. C2C12 cells were plated at low density (1.5×10^6) or high density (1.5×10^6) to produce myoblasts or myotubes respectively. Each precipitate contained 20 μg of test plasmid and 5 μg of CMV–β-gal plasmid. The day after plating, the cells were refed and incubated for 3 h. The cells were then incubated with precipitate for 4 h and subjected to a 3 min glycerol shock. The cells were then washed and refed. Cells were harvested in 72 h for assay, except for the C2C12 high-density cells, which were refed with DMEM/10% horse serum and harvested when fusion of myotubes was noted at 72–96 h. For co-transfection experiments with GATA-4, the calcium phosphate method was used. The precipitate contained 15 μg of test plasmid, 5 μg of either pMT+GATA-4 or pMT− and 5 μg of CMV–β-gal plasmid per 60 mm plate. COS cells were transfected with 25 μg of either pMT+GATA-4 or pMT− per 100 mm plate.

Assays

On the day of harvesting the cells were washed in Ca^2+/-free Mg^2+/-free PBS, and then lysed in 200 μl of 1% Triton lysis buffer (Analytical Luminescence). Untransfected cells were used as the blank for luciferase and galactosidase assays. Luciferase assays were performed on 20 μl of cell extract using the assay kit produced by Analytical Luminescence. Luminescence was measured in an Analytical Luminescence luminometer over 10 s. Samples that exceeded the linear range of the assay were diluted immediately and remeasured. To normalize between plates, β-galactosidase activity was measured in a 50 μl volume extract using standard methods [23].

Preparation of extracts

Nuclear extracts were prepared from neonatal rat hearts using the method of Dignam [24] with minor modifications as described [25]. Extracts from COS cells transfected with pMT+GATA-4 or pMT− were prepared by washing the cells, then scraping them into 25 μl per plate of 2× binding buffer (40 mM Hepes/NaOH, pH 7.8, 100 mM KCl, 20% glycerol, 1.0 mM EDTA, 1.0 mM MgCl₂), and taken through three cycles of freeze–thaw lysis.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as previously described [25]. The sense and antisense strands of the oligonucleotides used are as noted in Table 1. BNP spans a sequence from −103 to −80 of the rat B naturetic peptide gene [26]. TnIG spans −678 to −652 of the rat cardiac TnI gene and β2 spans a TEF-1-binding site from −288
to −267 of the rat β-myosin heavy chain (MHC) gene [25]. Additional oligonucleotides from the rat cardiac TnI gene sequence include: TnIG 750 which contains a GATA motif at −750 on the sense strand and spans from −760 to −735; TnIG 557 which contains a GATA motif at −557 on the antisense strand and spans from −568 to −545; TnIG 62 which contains a GATA motif at −62 on the antisense strand and spans from −75 to −50; TnIG 2 which contains a GATA motif at −2 on the antisense strand and spans from −14 to +11. The oligonucleotides were generally synthesized with BamHI/BgIII overhangs except as noted in Table 1. Oligonucleotides were synthesized with mutations as noted in Table 1. For competition studies a 200-fold molar excess of unlabelled competitor was used. Approx. 4–6 µg of extract was used per lane, and the results were analysed on a 6% polyacrylamide gel.

RESULTS

Gene structure and putative regulatory motifs

The TnI gene structure and DNA sequence derived from the rat genomic clones are shown in Figure 1. The gene has eight exons. Exon 2 is only 13 bp in length. In contrast with the avian fast skeletal-muscle TnI gene and the human slow skeletal-muscle TnI gene, the first exon of the cardiac TnI gene contains the start codon. The avian fast skeletal-muscle, rat and human slow skeletal-muscle TnI genes, like several other muscle-specific genes, have a first exon that is untranslated [12,13,27,28]. The intron-exon structure of the avian fast skeletal-muscle, the human slow skeletal-muscle and rat and mouse cardiac genes are completely conserved for exons 5, 6 and 7, which encode the majority of the protein sequence [13,29]. The structure and boundaries of exon 4 are also conserved between the slow skeletal-muscle and cardiac genes [13,29]. Exon 8 includes the stop codon and untranslated region in the fast skeletal-muscle and cardiac genes; however, the slow skeletal-muscle gene contains its 3′-untranslated region on a separate ninth exon [29].

The unique N-terminus of the cardiac TnI gene is encoded on exons 1, 2 and 3. Exon 3 includes the sequence for the functionally important serines which are phosphorylated by protein kinase A [30]. The ‘inhibitory’ region of the protein (amino acids 129–149 in the rat cDNA sequence), which as a peptide is capable of inhibiting actomyosin ATPase activity [31], is contained within exon 7 in all members of the gene family. The cysteines that interact with troponin T (TnT) [32] are split between exons 5 and 6.

In order to look for putative cardiac regulatory elements, computer searches of the 5′ regions of the rat cardiac TnI gene were performed. No homology was found with the cardiac regulatory motif HF-1 described by Zhu et al. [33], or the region said to confer cardiac-specificity on the cardiac TnT gene [34]. There was also no MEF-2 (YTAAAAATAACYYY) consensus site [35], although an AT-rich motif (AAATAAA) was found at position −538. No M-CAT (CATTCTT) motif [36], CARG box (CCWWWGWWGG) [37], β2 motif (TGTTGAATGT) [25], AP-1 site (STGACTVA) [38] or CAAT box was found. There are E-boxes at positions −435, −380 and −283, and CACCC motifs at positions −690, −470 and −128 on the antisense strand and −73 on the sense strand. Expression of TnI mRNA is influenced by thyroid status in neonatal rat pups [39]. The cardiac TnI mRNA content was approximately twice as high in euthyroid rat pups as in hypothyroid rat pups. Computer searches of the 1 kb upstream region revealed three thyroid-responsive element half-sites at positions −545, −42 and +51. The lack of complete palindromes makes it unlikely that these sites are transcriptionally active. Five GATA motifs were found in the upstream sequence at −750 on the sense strand and −658, −557, −62 and −2 on the antisense strand. Only the motif at −2 meets the requirements for a consensus GATA motif [40].

Table 1  Nucleotide sequence of sense and antisense strands of oligonucleotides used in EMSA

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td>5′-GATGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>β2</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG mut 670,664,659</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG mut 670,659</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG mut 664*</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG mut 670,664,659</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG 750</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG 557</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG 62</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG 2</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
<tr>
<td>TnIG 2 mut*</td>
<td>5′-GATCGGAGATGACGATAAGCAGATCCCA</td>
<td>5′-GATCAGGTATCTGGATTAGTAGCAGAC3′</td>
</tr>
</tbody>
</table>
A. M. Murphy and others

Figure 1

The TATA box at bp –31 is underlined. The cap site, as determined by primer extension analysis, is indicated in bold and underlined. Exons are displayed in upper case, and intervening and upstream sequence in lower case. Splice sites are underlined and in bold. The translated amino acid sequence is indicated by the single amino acid code on the line underneath the DNA sequence.

These sequence data are available from GenBank/EMBL/DDBJ under accession number U77354.

Regulation of cardiac-specific expression

Transient transfection experiments were used to examine upstream regions of the cardiac TnI gene which regulate cardiac specificity. TnI–luciferase reporter gene constructs were used to transfet cell lines as well as primary cardiocytes. A schematic of the reporter gene plasmids is illustrated in Figure 2. The results of these assays are compiled in Table 2. All of the TnI–luciferase constructs were preferentially active in cardiocytes, but the 896TnIpGL construct, in particular, demonstrated high levels of luciferase activity, over 7-fold that of the positive control vector pGL3 Control and 33-fold the activity in the hepatoma cell line HepG2. All other cell types, including the myogenic line C2C12, demonstrated relatively low luciferase activity. The 2000TnIpGL exhibited lower activity than 896TnIpGL,

suggesting that there may be a suppressor in this region. The region that contributes most strongly to positive transcriptional regulation (896 to 525) did not contain the E-box motifs, or other known cardiac regulatory motifs, although this region did contain three GATA motifs at positions 750, 658 and 557 and GATA-like motifs (GATT) at positions 669 and 665 (Figure 2).

GATA-4 activates cardiac TnI–luciferase in non-cardiocytes

Because GATA-4 has recently been identified as a cardiac transcription factor [19], its ability to trans-activate the cardiac TnI gene was examined. After trials at different ratios of GATA-4-expressing vector to reporter vector, a ratio of 1:3 GATA-4 to luciferase vector was found to be optimal. As illustrated in Figure 2.
Figure 2  Map of upstream region of the rat cardiac TnI gene and TnI–luciferase chimaeric plasmids

At the top is a map of the upstream region of the gene with the positions of the GATA, CACCC, E-box and TATA motifs indicated. Chimaeric plasmids with TnI upstream regions cloned into a luciferase (LUC) expression plasmid are illustrated below the schematic of the TnI gene. Control plasmids used in the transfection experiments include pGL2Basic which does not contain a promoter or enhancer and pGL2Control which contains a simian virus 40 (SV40) promoter and enhancer.

Table 2  Relative luciferase levels in cells transfected with cardiac TnI–luciferase fusion plasmids

Results are expressed relative to pGL2Control and are means ± S.E.M. for three to five independent transfections in which all plasmids were tested in parallel. They are normalized for β-galactosidase activity.

<table>
<thead>
<tr>
<th>Luciferase activity (relative to pGL2Control)</th>
<th>-150TnIpGL2</th>
<th>-524TnIpGL2</th>
<th>-896TnIpGL2</th>
<th>-2000TnIpGL2</th>
<th>pGL2 Basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiocytes</td>
<td>1.9 ± 0.52</td>
<td>3.54 ± 1.78</td>
<td>7.21 ± 1.92</td>
<td>3.65 ± 0.35</td>
<td>0.099 ± 0.004</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.39 ± 0.10</td>
<td>0.57 ± 0.10</td>
<td>0.22 ± 0.10</td>
<td>0.72 ± 0.16</td>
<td>0.001 ± 0.0003</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>0.18 ± 0.04</td>
<td>0.17 ± 0.04</td>
<td>0.17 ± 0.03</td>
<td>0.19 ± 0.05</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>C2C12MT</td>
<td>0.23 ± 0.07</td>
<td>0.35 ± 0.11</td>
<td>0.48 ± 0.07</td>
<td>0.45 ± 0.14</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>C2C12MB</td>
<td>0.78 ± 0.23</td>
<td>1.9 ± 1.4</td>
<td>1.4 ± 0.54</td>
<td>0.97 ± 0.50</td>
<td>0.017 ± 0.003</td>
</tr>
</tbody>
</table>

Table 3  Effects of co-transfection of pMT2-GATA-4 on luciferase activity in NIH 3T3 cells

The results are expressed relative to the positive control plasmid pGL2Control and are means ± S.E.M. for three experiments. They are normalized to β-galactosidase activity.

<table>
<thead>
<tr>
<th>Luciferase activity (relative to pGL2Control)</th>
<th>-150TnIpGL2</th>
<th>-524TnIpGL2</th>
<th>-896TnIpGL2</th>
<th>-2000TnIpGL2</th>
<th>pGL2 Basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT2-GATA-4</td>
<td>2.9 ± 0.76</td>
<td>4.37 ± 1.53</td>
<td>11.0 ± 2.07</td>
<td>5.33 ± 1.14</td>
<td>0.036 ± 0.0014</td>
</tr>
<tr>
<td>pMT2</td>
<td>0.38 ± 0.12</td>
<td>1.11 ± 0.35</td>
<td>1.68 ± 0.89</td>
<td>0.83 ± 0.09</td>
<td>0.005 ± 0.005</td>
</tr>
</tbody>
</table>

Table 3, co-expression of GATA-4 in the NIH 3T3 cells resulted in a striking increase in the reporter gene activity in these cells relative to the control vector. The relative luciferase activity obtained in these co-transfected NIH 3T3 cells is comparable with that obtained in cardiocytes. This activation is most notable with the -896TnIpGL2 construct which has 11-fold the activity of the pGL2Control vector, although the overall 6–7-fold increase compared with co-transfection with the control pMT2 vector was similar for the -150TnIpGL2, -896TnIpGL2 and -2000TnIpGL2 plasmids. The -524TnIpGL2 construct demonstrated only a 3.9-fold increase when compared with co-transfection with the control pMT2 plasmid.
A. M. Murphy and others

Figure 3 Binding of the TnI probe containing three GAT(A/T) motifs to nuclear protein from neonatal rat cardiac muscle and to GATA-4 expressed in COS cells

Labelled TnI (TnIG) probe was incubated with no extract (first lane), neonatal cardiac nuclear extract (lanes 2–5), extract from COS cells transfected with pMT2-GATA-4 (lanes 6–9) or COS cells transfected with the pMT2 vector alone (lane 10). Competitions were performed with 200-fold molar excesses of unlabelled self (TnIG), the BNP gene probe containing paired GATA sites or an unrelated probe from the β-MHC gene (βe2). The presence of either the cardiac nuclear extract or GATA-4 extract results in a gel shift of very similar mobility. The complex competes with the TnI probe or the BNP probe, but not with the βe2 probe.

GATA-4 binds specifically to GAT(A/T) motifs in the cardiac TnI sequence

An element from position −657 to −674 of the cardiac TnI gene contains a GATA motif and a GATA-like motif (GATT) in tandem on the antisense strand with spacing identical with tandem GATA motifs on the rat BNP gene [26]. These motifs are illustrated in Table 1 which specifies the oligonucleotides used in EMSA. There is also a GATT motif on the sense strand of this cardiac TnI element which lies between the two antisense GAT(A/T) motifs. These motifs are contained within the −896TnIpGL# construct which has the highest transcriptional activity in cardiocytes. EMSA was performed to assess DNA-binding activity to this region in cardiocytes and to examine whether GATA-4 expressed in COS cells would specifically bind to this probe. Nuclear extract from neonatal rat heart results in a gel shift of the TnI probe as illustrated in Figure 3. Binding activity was competed for by either the TnI oligonucleotide (TnIG) or oligonucleotide from the rat BNP gene (Table 1). An unrelated oligonucleotide from the rat β-MHC gene (βe2) did not compete. Extract from COS cells transfected with pMT2-GATA-4 complexed with the TnIG probe with the same gel-shift pattern. This activity was also competed for by either the TnIG or BNP probe. Extract from COS cells transfected with pMT2 did not result in a gel shift. These data indicate that this element of the cardiac TnI gene, which contains GATA and GATA-like motifs, was able to complex specifically with GATA-4.

The effects of mutations of single bases in the GAT(A/T) sites in the TnIG probe were analysed in Figure 4. An oligonucleotide containing mutations in all three GAT(A/T) motifs did not compete for binding to the probe. An oligonucleotide containing mutations in either flanking antisense motif and the sense motif did not compete for GATA-4 DNA binding. Oligonucleotides in which either both flanking antisense motifs or the sense GATT motif were intact were capable of competing GATA-4 DNA binding. The oligonucleotides which contained a single intact antisense motif did not compete for GATA-4 DNA binding.

Additional GATA sites complex with GATA-4 in vitro

The putative GATA DNA-binding sites at positions −750, −557, −62 and −2 were assessed by performing competition
Figure 5  Binding of additional GATA sites in the TnI upstream sequence to GATA-4 in vitro

Labelled TnI (TnIG) probe was incubated with either no extract (first lane) or extract from COS cells transfected with pMT2-GATA-4 (lanes 2–7). Competitions were performed with 200-fold molar excesses of unlabelled self (TnIG), an oligonucleotide with GATA site at —750 on the sense strand (TnIG 750), an oligonucleotide with a GATA site at —557 on the antisense strand (TnIG 557), an oligonucleotide with the GATA site at —62 on the antisense strand (TnIG 62) and an oligonucleotide with the GATA site at —2 on the antisense strand (TnIG 2). TnIG 750 and TnIG 2 compete for the binding of GATA-4 to the TnIG probe, whereas TnIG 557 and TnIG 62 compete weakly if at all.

Figure 6  Binding of an antisense GATA-4 site at bp —2 with GATA-4

Labelled TnI probe with the GATA site at bp —2 (TnIG 2) was incubated with no extract (first lane), extract from COS cells transfected with pMT2-GATA-4 (lanes 2–6) or COS cells transfected with the pMT vector alone (lane 7). Competitions were performed with 200-fold molar excesses of unlabelled self (TnIG2), a probe containing a cluster of GATA sites at —669/665/658 (TnIG), the BNP gene probe containing paired GATA sites, an unrelated probe from the β-MHC gene (βe2) and a TnIG 2 probe with a single mutation (GATA to GGTA). The TnIG 2 probe complexes with GATA-4 and this interaction is competed for by self, by the GATA-binding probes TnIG and BNP, but not by the non-specific probe βe2 or by a probe containing the mutated GATA site.

EMSAs. The results, illustrated in Figure 5, demonstrated the ability of the unlabelled probes containing GATA sequences at —750 and —2 to compete for binding of GATA-4 to the TnIG probe containing three GAT(A/T) motifs. The ability of the TnI —2 GATA site to specifically bind GATA-4 was further assayed by labelling this probe and performing competition studies with specific and non-specific competitors as well as with a probe containing a single mutation in the GATA site (GATA to GGTA). The results are illustrated in Figure 6. There is specific binding of the GATA-4-containing cell extract to the —2 GATA motif, and the single mutation resulted in loss of the ability of this oligonucleotide to compete for GATA-4 DNA binding.

A mutation in the proximal GATA-4 site results in reduction of transcriptional activation

Transient transfection assays were performed on constructs containing the mutation that resulted in loss of competition of GATA-4 DNA binding to the —2 GATA site in the EMSA.

Table 4 Results of transient transfection experiments with TnI–luciferase reporter gene constructs containing a mutation in the proximal GATA-binding site

<table>
<thead>
<tr>
<th>Luciferase activity (relative to pGL2Control)</th>
<th>−150TnIpGL2</th>
<th>−896TnIpGL2</th>
<th>pGL2 Basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>−150TnIpGL2 mut</td>
<td>0.32±0.29</td>
<td>0.06±0.08</td>
<td>0.09±0.13</td>
</tr>
<tr>
<td>−896TnIpGL2 mut</td>
<td>11.78±5.64</td>
<td>0.09±0.13</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.42±0.93</td>
<td>0.06±0.08</td>
<td>0.09±0.13</td>
</tr>
</tbody>
</table>

Results in these functional assays demonstrate that this mutation reduces the luciferase activity of the −150TnIpGL2 construct by 80% in the transfected cardiocytes (Table 4). This effect was present in both the −150TnIpGL2 and the −896TnIpGL2
construct, indicating that an intact proximal GATA site is necessary to support transcriptional activity in cardiocytes.

**DISCUSSION**

This work demonstrates that the proximal promoter region of the rat cardiac TnI gene is sufficient to support high levels of reporter gene activity in cardiocytes and demonstrates relatively low activity in non-muscle and skeletal-muscle cell lines. In this regard, the cardiac TnI gene differs from the fast and slow skeletal-muscle family members, which like other muscle genes, require intronic regions to mediate high levels of expression [12–15]. The finding that the proximal upstream region supports high levels of reporter gene activity in cardiocytes is not unexpected given the reports of other investigators noting that the cardiac-specific regulation of genes, such as myosin light chain 2 (MLC-2) and troponin C (TnC), may be mediated by immediate upstream regions [33,41]. This study is limited in that the contribution of 3’ regions of the cardiac TnI gene have not been assessed.

Although it is difficult to compare studies utilizing different reporter genes, this report contrasts with that of Ausoni et al. [29], who reported relatively poor activity of similar constructs of the mouse TnI gene in their transient transfection experiments. These authors noted that mouse cardiac TnI–chloramphenicol acetyltransferase (CAT) constructs were at most capable of supporting activity that was an order of magnitude less than pSV3CAT. In the present experiments the cardiac TnI–luciferase constructs exceeded the activity of the pGL-Control by up to 7-fold. It is possible that the relatively lower activity reported for transcriptional assays with the mouse promoter was due to the use of constructs in which the promoter was truncated a few base pairs downstream from the conserved proximal GATA site [29]. Furthermore, no specific regulatory elements in the mouse cardiac TnI promoter were defined in that study.

The results of these studies suggest that GATA-4 plays an important role in the regulation of the cardiac TnI gene. GATA-4 has recently been shown to be expressed in the precardiac mesoderm in the embryonic mouse heart starting at postcoital day 7.0 to 7.5, at day 8 in the developing heart tube and in rat neonatal cardiomyocytes [42,43]. Thus its developmental pattern of expression temporally precedes that of cardiac TnI mRNA in rodent heart, suggesting that GATA-4 could mediate the developmental up-regulation of this gene [4,44]. GATA-4 may also mediate effects of thyroid hormone on TnI expression. We had previously demonstrated that neonatal rats that were made hypothyroid had delayed switching of TnI isoforms [39]. Molkentin et al. [45] have demonstrated that exogenous thyroid hormone up-regulates GATA-4 DNA-binding activity 3-fold in neonatal cardiocytes, possibly explaining the delay in up-regulation of cardiac TnI mRNA in hypothyroid neonatal rat hearts.

Although other cis-acting cardiac regulatory elements have been characterized, GATA motifs in the α-MHC gene, the TnC gene and the rat BNP gene have been demonstrated to interact specifically with GATA-4 [26,43,45]. In the rat BNP gene, there is a proximal GATA-4 DNA-binding site at −30, as well as paired GATA-4 DNA-binding motifs at −90. Mutation of both of the paired upstream GATA motif results in a 10-fold reduction in reporter gene activity of BNP constructs [26]. Mutation of the GATA site at the −30 position was not functionally assessed [26]. In addition, the presence of the paired upstream GATA motifs from the BNP gene activated a heterologous promoter in cardiocytes [26]. Expression of GATA-4 in non-muscle cell lines was demonstrated to activate reporter gene constructs of the BNP and TnC genes; however, GATA-4 was only capable of trans-activating the α-MHC reporter gene constructs in a skeletal-muscle context [26,43,45].

Despite some similarities in the regulation of cardiac contractile protein genes by GATA-4, the developmental patterns of expression differ between TnI, TnC and α-MHC genes. TnC is the predominant isoform in heart throughout development, although there is transient expression of fast skeletal-muscle TnC in embryonic heart [46]. α-MHC is expressed in embryonic mouse heart tube between day 7.5 and 8 post coitum, but β-MHC predominates in fetal rodent heart [47]. Cardiac TnI mRNA is not detectable until a slightly later stage in rodent heart, and is preceded by expression of the slow skeletal-muscle TnI isoform in heart [44]. Although α-MHC is rapidly induced after birth in the rodent heart, cardiac TnI is not fully expressed until weaning [4]. Furthermore α-MHC is a minor isoform in human cardiac ventricle, whereas TnI isoform switching is highly similar between rodent and human heart [8]. Two recent studies have explored the role of GATA-4 in early cardiocyte differentiation. Grepin et al. [48] have suggested that GATA-4 plays a role in cardiac cell commitment, as evidenced by the lack of cardiocyte differentiation in P19 cells transfected with antisense GATA-4 constructs; however, data from chimaeric mice created by blastocyst injection with embryonic stem cells with GATA-4 deleted indicates that these cells have the capacity to contribute to the myocardium [49]. By analogy with the roles of GATA and other transcription factors in haematopoietic lineages [50], GATA-4 and other GATA proteins and cofactors in the heart may play a role in both early cardiocyte differentiation, as well as later stages of developmental regulation. In this regard the TnI gene family may be an excellent model system for dissecting the role of cardiac transcription factors at mid to late gestation as well as early postnatal life.

To date relatively few cardiac-restricted transcription factors have been defined. Other cardiac gene regulatory factors, such as TEF-1 and the MEF-2, are expressed in a variety of tissues, but DNA-binding activity is found predominantly in both forms of striated muscle [51–54]. Although GATA-4 is expressed in the cardiac but not skeletal-muscle lineage, it is not strictly cardiac-specific in that it is expressed in gut and gonadal tissues. Additional GATA family members have been defined in the chicken and rat, which have overlapping but distinct patterns of expression in heart and gut [55,56]. In the haematopoietic system, GATA factors may self-interact [57], interact with or regulate other GATA factors [57,58] or interact with ubiquitous or tissue-specific factors [59]. Similar complex interactions are likely to take place in the cardiovascular system.

Taken together these data indicate that the proximal regions of the cardiac TnI gene regulate its cardiac-specific expression. Comparison of the regulatory regions of cardiac TnI with that of the fast and slow twitch skeletal-muscle TnI genes follows the pattern found in other contractile protein genes in which skeletal-muscle enhancers are located in intronic regions whereas those mediating high levels of cardiac expression are located in the proximal upstream regions. A proximal GATA-4-binding site in the cardiac TnI gene is necessary for the transcriptional activation of this gene in vitro, and other sites of GATA-4 DNA binding may contribute to the regulation of this gene.

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