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

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Troponin I is a thin-filament contractile protein expressed in striated muscle. There are three known troponin I genes which are expressed in a muscle-fibre-type-specific manner in mature animals. Although the slow skeletal troponin I isoform is expressed in fetal and neonatal heart, the cardiac isoform is restricted in its expression to the myocardium at all developmental stages. To study the regulation of this cardiac-specific and developmentally regulated gene *in vitro*, the rat cardiac troponin I gene was cloned. Transient transfection assays were performed with troponin I-luciferase fusion plasmids to characterize the regulatory regions of the gene. Proximal regions of the upstream sequence were sufficient to support high levels of expression of the reporter gene in cardiocytes and relatively low levels in other cell types. The highest luciferase activity in the

cardiocytes was noted with a plasmid that included the region spanning –896 to +45 of the troponin I genomic sequence. Co-transfection of GATA-4, a recently identified cardiac transcription factor, with troponin I-luciferase constructs permitted high levels of luciferase expression in non-cardiac cells. Electrophoretic mobility-shift assays demonstrated specific binding of GATA-4 to oligonucleotides representative of multiple sites of the troponin I sequence. Mutation of a proximal GATA-4 DNA-binding site decreased transcriptional activation in transfected cardiocytes. These results indicate that the proximal cardiac troponin I sequence is sufficient to support high levels of cardiac-specific gene expression and that the GATA-4 transcription factor regulates troponin I-luciferase expression *in vitro*.

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 natriuretic 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 pGL₂Basic (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 –150TnIpGL₂ (–150 to +51) was constructed by digesting the 1.2 kb genomic subclone with *Apal* (–150) and *BstEII* (+51), blunting with T4 polymerase, subcloning this into digested blunted pGEM-3Z vector, and subsequently placing the insert directionally into pGL₂Basic digested with *SmaI* and *SacI*. The construct –524TnIpGL₂ (–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 blunted pGL₂Basic. 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 pCRTMII (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 –896TnIpGL₂ (–896 to +45) clone was prepared by digesting this chimaeric 1.2 kb clone with *HindIII* and *EcoRI*, blunting and cloning into *XhoI*-digested blunted pGL₂Basic. Orientation was confirmed by sequencing. The plasmid –2000TnIpGL₂ was prepared by digesting –896TnIpGL₂ with *KspI* (–859) and *SacI* (in the 3' linker of pGL₂Basic) and ligating an upstream 1 kb *SacI*–*KspI* fragment from the 3 kb *EcoRI* genomic subclone. Additional plasmids were purchased for use including pGL₂Control (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 pMT₂-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 TransformerTM 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 μ g of test plasmid and 5 μ g of CMV– β -gal were added to 50 μ l 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 (1.5×10^5) or high density (1.5×10^6) to produce myoblasts or myotubes respectively. Each precipitate contained 20 μ g of test plasmid and 5 μ g 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 μ g of test plasmid, 5 μ g of either pMT₂-GATA-4 or pMT₂ and 5 μ g of CMV– β -gal plasmid per 60 mm plate. COS cells were transfected with 25 μ g of either pMT₂-GATA-4 or pMT₂ per 100 mm plate.

Assays

On the day of harvesting the cells were washed in Ca²⁺-free Mg²⁺-free PBS, and then lysed in 200 μ l 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 μ l 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 μ l 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 pMT₂-GATA-4 or pMT₂ were prepared by washing the cells, then scraping them into 25 μ l per plate of $2 \times$ 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 natriuretic peptide gene [26]. TnIG spans –678 to –652 of the rat cardiac TnI gene and β e2 spans a TEF-1-binding site from –288

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

GAT(A/T) motifs on either sense or antisense strands are noted in bold. Mutated bases are underlined. Oligonucleotides were synthesized with *Bgl*II/*Bam*HI overhangs except as noted by the asterisk.

Oligonucleotide	Sense	Antisense
BNP	5'-GATCTGTCT GATA AATCAGAGATAACCCA	5'-GATCTGGGTATCTCT GATT TATCAGACA-3'
β e2	5'-GATCTGTGAGCTGTGAATGTAAGGGA	5'-GATCTCCCTTACATTCCACAGCTCACA-3'
TnIG	5'-GATCGATCATAATCTAA GATT TATCCTGACA	5'-GATCTGTCAG GATA AATCTTA GATT ATGATC-3'
TnIG mut 670,664,659	5'-GATCGATCATAACCTAA GGTT ACCTGACA	5'-GATCTGTCAG GGTAA ACCTTAG GGTT ATGATC
TnIG mut 670,659	5'-GATCGATCATAACCTAA GATT ACCTGACA	5'-GATCTGTCAG GGTAA ATCTTA GGTT ATGATC
TnIG mut 664*	5'-GATCATAATCTAA GGTT TATCCTGACA	5'-TTCTTGTGAG GATA AACCTTAG GATT ATG
TnIG mut 670,664	5'-GATCGATCATAACCTAA GGTT TATCCTGACA	5'-GATCTGTCAG GATA AACCTTA GGTT ATGATC
TnIG mut 664,659	5'-GATCGATCATAATCTAA GGTT ACCTGACA	5'-GATCTGTCAG GGTAA ACCTTA GATT ATGATC
TnIG 750	5'-GATCAACCCAGT GATA AACTAGAATT	5'-GATCAATTCTAGTTATATCACTGGGGTT
TnIG 557	5'-GATCCAAGGACATATCCTCCCTGGATG	5'-GATCCATCCAAGGGAG GATA TGTCTTG
TnIG 62	5'-GATCCACCTGTTATCGCTCATCTGGG	5'-GATCCCCAGGATGAG GATA ACAGGGTG
TnIG 2	5'-GATCTGCCCCCTTATCTCAGTGCCTCA	5'-GATCTGAGGACACTG GATA AGGGGCGA
TnIG 2 mut*	5'-TCCTCGCCCTTACCTCAGTGCTCTCA-3'	5'-TGAGGACTGAG GGTAA AGGGGGGAGGA

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 bases -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 *Bam*HI/*Bgl*II 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 μ g 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 (CATTCCT) motif [36], CArG box (CCWWWWWWGG) [37], β e2 motif (TGTGGAATGT) [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].

-896	tgccaatgtc	ctcggcgatt	gcacctctaa	aacatgtaac	atgaaccgag	1405	GAGCACGCGT	TGCCAGCCCT	TGGTGTGGA	TGGGCTGGGC	TTTGAAGAGC
-846	gggaccaaga	atcctacctt	taactgtgta	agcaggctcc	aggactgaca		S T R C Q P L V L D G L G F F E E				
-796	ccagctcagc	ccactgtttt	cctctcgcgt	cctctcgcgt	cccagtgata	1455	TTCAAGTatc	agttttaccaa	gcagggaattc	ctgatgagcc	tgggtccga
-746	taactagaat	ttcaaatctc	ttctcttgaa	ctctcagttc	tagtcttttt		L Q				
-696	tggtgtgtgt	gtgtctctaga	tcataactcta	agatttatcc	tgacaagaat	1505	tgtttctcat	ctcggggcccg	gcactctcttg	ggatcaggga	gcacaagaac
-646	cccaggccag	aggtacaacc	ctgggtctcca	catcctcttt	tgcttggggc	1555	catccccagt	tctgtctcagg	atctgcaatc	cttatgcaaa	gctatacatt
-596	ctttcttgac	atgctactgt	tggaagggaca	aggacatatc	ctcccttgga	1605	cagtcttaca	attcaaaatc	cagtgatatt	ttcctgcctt	tgctgtctaa
-546	tgcggttcaa	ataaagagcg	cagggtcacc	tctgtgtgtc	ctgtcctctg	1655	gtctcagaat	tcttggctcc	tgcccatatt	tttttttaag	ttccctagag
-496	ttggtctcca	gtgccaagaa	gggggttgga	caagggggga	tgggcccccg	1705	tgcggtatcc	agtgtcagct	tcctttcacc	ccctcctctc	tcagactcag
-446	atggtgggtt	tccaagtgga	cttggtcgaa	ttccacatca	gtgtaggctg	1755	gagtcacagac	cccagcactc	ctctctcaaa	ctcaggggtc	cagagcctag
-396	aggacttagg	gtcctgcaca	tgaaccttta	aactaaacat	gctaagggca	1805	gcctccttcc	tcagaccggg	agggtctcct	tctcagatca	gagacctgag
-346	gtgaaactga	cagaaacatg	gagagaaaag	tcacagaaaag	actacatttt	1855	tctccagcct	tctctcaca	gcccatgcat	tctctcaca	gGACCTATGC
-296	atgtaggaac	ctacatgtga	ggaaaagaaga	aggcctggat	ttttgggttaa						D L C
-246	tctcacagga	aactgaagtc	tggactcttg	gatttgagag	aagagggacc	1905	CGGCAGCTTC	ACGCTCGTGT	GGACAAAGTG	GATGAAGAGA	GATACGACGT
-196	ttgtctactg	gttttctctga	gtctaaagga	gaaggggagct	ggggccccaga		R Q L H A R V D K V D E E R Y D V				
-146	gtcaaaagga	ggaggggtgt	agatccgggc	accttgggtg	gtctaactgg	1955	GGAAGCAAAA	GTCACCAAGA	ACATCATCTGA	Ggtgggaagc	R Y Y daagt
-96	agctttgcac	acggctcccc	tcacaccctg	ttatcgctca	tcctgggaag		E A K V T K N I T E				
-46	gggaggagac	agcagtatat	ttagtctttg	tctctgcccc	ttatctCAGT	2005	ctgagcacct	tcactgtaga	ccgagatgtc	caggactgta	gcttcagact
5	GTCTCAGTG	AGGCTTGAG	AGCAGCAGG	GGACCCAAG	CTCCAAGGTC	2055	gagggctatg	tctgtgtgta	cttttagaca	ctctaagcag	aaggcaggca
55	ACCAGGACA	CCCTTCTAAG	ACCTCCAGG	AATCTGCAAT	CCCATCTCT	2105	gtggcaagg	cagctgttcc	cacagagaga	caacacgtgc	aaggtagaac
105	ACCTCTGGAG	ATCAGCATGG	CGGATGAGta	tgtgaacctt	ggtaaccttg	2155	ctgggtgggt	taaatcggtc	tgtagaaact	gagcactgag	ttgggtctctg
		M A D E				2205	ggaataatga	tctgcacatc	attggggccag	ctcgtgttag	aggctttgat
155	aactggagac	cccaaggaac	acgggtgcagt	cagccttcag	ggtccttctg	2255	tgatgggact	cggagtatca	ggaagagcac	aaagtgtgag	attggaccct
205	aggggtgaac	atggttgaat	tcttctcctg	agatggtagg	agggcaagggt	2305	ttaatctgga	gtcagacctg	gaagagagga	ggaaccttag	attctgagag
255	ggttggattt	ctgtagaat	gaggttaagaa	ttcggaata	gactcctggg	2355	caagataggg	ataaggggga	attgtttgtg	agaaaccttag	ggtagggagg
305	gtctaaggga	gtagaaaagg	agccccaacc	ttgttcaggga	ggactgtctgt	2405	aaacagaggg	aagacgcggt	ctcctcccc	catatgtggg	ttccccgATT
355	caatagactt	ctactctcac	ggtttctctc	ccttcagGAG	CAGCGATCGG						I
				S D A		2455	GCAGATCTGA	CCCAGAAGAT	CTATGACCTG	CGTGGCAAGT	TTAAGCGGGC
405	gtgagcactg	tgacctaaga	agtggggtag	ggaacccctg	gcccagagag		A D L T Q K I Y D L R G K F K R P				
455	ggtgctgcag	ggagaggatg	gggaccgatg	ggcaggggaa	tctgcagcta	2505	CACCTCTCCGA	AGAGTGAGAA	TCTCAGCAGA	TGCCATGATG	CAGGCACTAC
505	gaggtgagga	atccgggaag	gactggaggg	aggggtgggg	tagtaactgta		T L R R V R I S A D A M M Q A L				
555	gcctgcttca	atttcagGCT	GGGGAACCCC	AGCCGGCGCC	TGCTCCTGTC	2555	TGGGGACCCG	GGCCAAGGAA	TCCTTGGACC	TGAGGGCCCA	CCTCAAGCAG
		A G E P Q P A P A P V				2605	GTGAAGAAGG	AGGACATTGA	GAAGGtgagt	gtgggctagg	gtaagaagga
605	CGACGTCGCT	CCTCGGCCAA	CTACCGAGCC	TATGCCACCG	AGCCACATGC		V K K E D I E K				
655	CAAGtgggg	aggggtctag	aggacggtag	ttctgggttc	agaactcctc	2655	agatgcttgg	gaaggtctga	gtggccataa	ggtccagaca	gagagcagac
	K					2705	acatttgggg	ttctagttag	aagctacgga	taaaagggaac	tcaagaactc
705	gggaccccg	ggcgccacca	aggttggaa	gagaaactcc	tctgcctaca	2755	atgaaggagg	acagttcatt	aggagagaaa	gaagttagcaa	ggggcaggaa
755	gcaaggcata	ggcgtgacga	cagtgattag	ataggcagtg	ataggcagtg	2805	gtgcaaaagag	actggagggtg	catgagggag	acaagaagtg	cttggaggag
805	tgtagctcta	ctcagagaca	aagatttggtg	gatggggagc	aagacgtggc	2855	acaggaagtg	tatgggggtg	agtggagggtg	ctttagggaga	gtacagggagg
855	ttccagtggg	gtcagcattc	tggttagaag	aatgggtgag	cogaatggc	2905	gaaactggaa	gcacttggga	agactgcagg	tgcatggggg	tgactggaga
905	atgcacttag	agtgggtggt	ggtgccttgc	acccccgcc	aatgtccact	2955	tgcaagggga	aacaggaagt	acaagagggg	gacaggaagt	gcattggggc
955	ttgggacctc	tcactgacct	ttctccctat	cacctggcag	AAAAAGTCTA	3005	cactagaggt	gcacttggaa	gacagacatt	catgagaaag	actggagatg
					K K S	3055	agtggagggt	gcaggggagt	caggggctga	gtagagggtg	ataggggaga
1005	AGATCTCCGC	CTCCAGAAAA	CTTCAGTTGA	AGgtgatgaa	gagctcggag	3105	atagaggtgc	atgtggggaga	caagaaactc	atagtgtaga	caggcagtg
	K I S A S R K L Q L K					3155	atggggagac	aggaagtaca	tgggcaagac	aggaagtcca	tggggaagcc
1055	gggtgggctc	tggtgggccc	agcttttggg	gagtggtttc	gtttcggagt	3205	tggtatgacc	tgaggggaca	agaaatacat	gggggtacaa	gaagtgcacg
1105	gcgtcagccc	ccctacacac	acacacacac	acacacacac	acacacacac	3255	ggggagactg	gaagtgccta	gggggtgact	gaggtacccc	ggaagtgggg
1155	acacacacgt	gcgcgtgcac	acacacagaa	aacgggggaa	gttttttttt	3305	aagtattaca	gggagtttcc	caggtgtgtc	gaggagacag	gaagcattct
1205	ttataaaaag	acacaaaact	ggatgttttg	atatctctgtc	tctctggaga	3355	agaaccagat	tcttagatag	gcttccggca	cagaaaggcc	taggaaacta
1255	atagcgagat	agagggaaat	ttgttattct	tatactttgt	gggacctgtg	3405	gaagacagac	agaaaacgtt	cagggtataa	agaccagaaa	gaattgttaa
1305	tttcggctcc	caccattccg	cggACTCTGA	TGCTGCAGAT	TGCGAAGCAG	3455	cctctgacct	tctacctcca	gGAAAACCGG	GAGGTGGGAG	ACTGGCGCAA
			T L M L Q I A K Q					E N R E V G D W R K			
1355	GAGATGGAGC	GTGAGGCAGA	GGAGCGACGT	GGAGAGAAGG	GCCGCTTCT	3505	GAATATCGAT	GCACAAAGTG	GAATGGAAGG	CCGAAAGAAA	AAATTTGAGG
	E M E R E A E E R R G E K G R V L					3555	GCTAGGCCCA	TGGCTCTCAC	ACTGTGCTCT	GAAGGGCATC	TCTGAGGAAT
						3605	AAATTTCTTT	AAACTGG			

Figure 1 Sequence of the upstream region and the rat cardiac TnI gene

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/DBJ 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 transfect 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 -896TnIpGL₂ construct, in particular, demonstrated high levels of luciferase activity, over 7-fold that of the positive control vector pGL₂Control and 33-fold the activity in the hepatoma cell line HepG2. All other cells types, including the myogenic line C2C12, demonstrated relatively low luciferase activity. The -2000TnIpGL₂ exhibited lower activity than -896TnIpGL₂,

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 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 $\pm$ S.E.M. for three to five independent transfections in which all plasmids were tested in parallel. They are normalized for β -galactosidase activity.

	Luciferase activity (relative to pGL ₂ Control)				
	—150TnIpGL ₂	—524TnIpGL ₂	—896TnIpGL ₂	—2000TnIpGL ₂	pGL ₂ Basic
Cardiocytes	1.9 $\pm$ 0.52	3.54 $\pm$ 1.78	7.21 $\pm$ 1.92	3.65 $\pm$ 0.35	0.009 $\pm$ 0.004
HepG2	0.39 $\pm$ 0.10	0.57 $\pm$ 0.10	0.22 $\pm$ 0.10	0.72 $\pm$ 0.16	0.001 $\pm$ 0.0003
NIH3T3	0.18 $\pm$ 0.04	0.17 $\pm$ 0.04	0.17 $\pm$ 0.03	0.19 $\pm$ 0.05	0.002 $\pm$ 0.001
C2C12MT	0.23 $\pm$ 0.07	0.35 $\pm$ 0.11	0.48 $\pm$ 0.07	0.45 $\pm$ 0.14	0.004 $\pm$ 0.001
C2C12MB	0.78 $\pm$ 0.23	1.9 $\pm$ 1.4	1.2 $\pm$ 0.54	0.97 $\pm$ 0.50	0.017 $\pm$ 0.003

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

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

	Luciferase activity (relative to pGL ₂ Control)				
	—150TnIpGL ₂	—524TnIpGL ₂	—896TnIpGL ₂	—2000TnIpGL ₂	pGL ₂ Basic
pMT ₂ GATA-4	2.9 $\pm$ 0.76	4.37 $\pm$ 1.53	11.0 $\pm$ 2.07	5.33 $\pm$ 1.14	0.036 $\pm$ 0.0014
pMT ₂	0.38 $\pm$ 0.12	1.11 $\pm$ 0.35	1.68 $\pm$ 0.89	0.83 $\pm$ 0.09	0.050 $\pm$ 0.005

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.

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 (β e2). 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 β e2 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 –896TnIpGL₂ 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 (β e2) 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

Figure 4 Competition EMSAs showing that GATA-4 may complex with either sense or antisense motifs in the –678 to –652 (TnIG) probe

Labelled TnI (TnIG) probe was incubated with no extract (first lane), extract from COS cells transfected with pMT₂-GATA-4 (lanes 2–8) or extract from COS cells transfected with pMT₂. Competitions were performed with 200-fold molar excesses of unlabelled self (TnIG), oligonucleotides containing mutations in all three GAT(A/T) motifs (670, 664, 659), mutations in the two flanking antisense GAT(A/T) sites (670, 659), a mutation in only the sense GATT site (664), mutations in the upstream antisense GATT and the sense GATT site (670, 664), and mutations in the sense GATT site and the downstream antisense GATA site (664, 659). Oligonucleotides in which either the sense GATT motif or both of the antisense GAT(A/T) motifs were intact compete for GATA-4 DNA binding.

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

Extract	-	GATA	GATA	GATA	GATA	GATA	GATA	GATA	PMT
Competitor	-	-	TnIG	TnIG mut 670 664 659	TnIG mut 670 659	TnIG mut 664	TnIG mut 664	TnIG mut 664 659	-

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.

EMSA. 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.

Extract		GATA	GATA	GATA	GATA	GATA	PMT
Competitor			TnIG 2	TnIG	BNP	TnIG 2mut

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 pMT₂ 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 (β e2) 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 β e2 or by a probe containing the mutated GATA site.

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

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

Luciferase activity (relative to pGL ₂ Control)					
	—150TnIpGL ₂	—150TnIpGL ₂ 2 mut	—896TnIpGL ₂	—896TnIpGL ₂ 2 mut	pGL ₂ Basic
Mean	1.42 $\pm$ 0.93	0.32 $\pm$ 0.29	11.78 $\pm$ 5.64	0.06 $\pm$ 0.08	0.09 $\pm$ 0.13

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 pSV₂CAT. In the present experiments the cardiac TnI–luciferase constructs exceeded the activity of the pGL₂Control 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 motifs 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 line 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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