Identification of cis Elements in the Cardiac Troponin T Gene Conferring Specific Expression in Cardiac Muscle of Transgenic Mice

Q. Wang, C.D. Sigmund, J.J.-C. Lin

Abstract—To investigate the underlying mechanism regulating cardiac gene expression, transgenic mice carrying the rat cardiac troponin T proximal promoter (~497 bp from the transcriptional start site) fused to a LacZ or chloramphenicol acetyltransferase (CAT) reporter gene were analyzed. The LacZ expression pattern throughout development was very similar to that of the endogenous cardiac troponin T gene. Within this promoter, a high degree of sequence homology was found at 2 sites, modules D (~335 to ~289 bp) and F (~249 to ~209 bp). Both regions contain at least a TCTG(G/C) direct repeat and an A/T-rich site, whereas only the F module has a muscle enhancer factor 2 (MEF2)-like motif. No significant decrease in CAT transgene expression was observed when only the MEF2 core sequence was mutated. However, when the MEF2 core sequence and its flanking TCTGG site were mutated (Mut5), CAT transgene expression was significantly decreased in the heart, and ectopic expression of the transgene was also observed. When mutations were introduced into this promoter to destroy all upstream TCTG(G/C) direct repeats in the D module (MutD), CAT expression remained cardiac specific, but the expression level was dramatically decreased. Relaxation of cardiac-specific transgene expression became even more severe in transgenic mice carrying double mutations (Mut[D+5]). In addition, CAT activity in the heart was nearly abolished. These results suggest that D and F modules have an additive function in determining the level of expression in the heart and only the F module confers cardiac-specific expression. (Circ Res. 2000;86:478-484.)

Key Words: transgenic mice ■ promoter ■ TCTG(G/C) direct repeat ■ A/T-rich region ■ cardiac-specific gene expression

The discovery of the MyoD family of proteins, which are members of the basic helix-loop-helix transcription factors, greatly advanced our understanding of the molecular mechanisms that regulate skeletal muscle differentiation.1–6 Although cardiac and skeletal muscle are both derived from mesoderm and express many of the same muscle genes, skeletal muscle basic helix-loop-helix proteins are not detected in the heart. Furthermore, mice lacking the MyoD family genes have normal beating hearts.7–9 Studies of heterokaryons between muscle cells and fibroblasts provide clear evidence that the cardiac muscle phenotype is recessive, in contrast to a dominant skeletal muscle phenotype.10 These results also suggest that cardiac myogenesis may occur through a combinatorial pathway, differing from the master-regulatory mechanism seen in skeletal myogenesis. Recent promoter studies of muscle protein genes11–15 have also documented that distinct cis-regulatory elements in these promoters are required for muscle specificity in cardiac versus skeletal muscle. Thus, the question remaining is how cardiac-specific gene expression is controlled.

As development proceeds, rat cardiac troponin T (cTnT) mRNAs can be detected in the developing heart and in some developing skeletal muscles. However, the level of cTnT expression in developing skeletal muscle is much lower than that in the developing heart. This aspect is different from that for the chick cTnT gene.16 At late fetal stages, transcription of the rat cTnT gene is specifically repressed in developing skeletal muscle. The cTnT proteins detected in skeletal muscles progressively decline and disappear 2 weeks after birth.17 In the adult, cTnT is expressed only in cardiomyocytes. Thus, the rat cTnT gene is an excellent model for studying cardiac-specific gene expression and for defining cardiac-specific trans-acting factors. We have previously identified a cis-regulatory 41-bp region (F module) within the proximal ~497-bp promoter of the cTnT gene, which is sufficient to direct significant expression in cultured neonatal cardiomyocytes.18 In the present study, several site-directed mutants were generated and examined for their promoter activities in both cultured neonatal cardiomyocytes and transgenic mice. Results suggest that 2 sequence homologous modules, D and F, within this promoter are important in determining cardiac specificity and the level of transgene expression.

Materials and Methods

Site-Directed Mutagenesis

Site-directed mutagenesis was performed with plasmid pBCAT218 as a template using the Transformer Site-Directed Mutagenesis kit.
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The proximal promoter of the cTnT gene

Figure 1. Diagram of the rat −497-bp cTnT promoter. Both D and F modules contain at least 1 A/T-rich site and 1 TCTG(G/C) direct repeat. Relative positions of the TCTG(G/C) sequence are indicated by arrows. The A/T-rich site in the F module is identical to that found in the D module. The F module contains another A/T-rich core sequence within a consensus MEF2 site (MEF2-like motif).

The use of laboratory animals in the present study was in accordance with guidelines approved by IACUC. Our institution has a current PHS animal welfare assurance.

pBcAT2 and the mutant constructs were used to generate transgenic mice as described.19 Transgenic founders were identified by PCR and dot-blot hybridization of tail biopsy DNA for the presence of CAT gene. The relative ratio of CAT to a single gene, caldesmon,20 in dot blots was used as a reference for comparison of transgene copy numbers. Transgenic and nontransgenic litters were weaned at 4 to 6 weeks, and euthanized, and tissues were homogenized in buffer A and analyzed for CAT activities.18 Total proteins in various tissues were determined by the Bradford method.21 Mann-Whitney tests22 were performed for data comparisons between transgenic and nontransgenic mice as described.19 Transgenic founders were identified by PCR and dot-blot hybridization of tail biopsy DNA for the presence of CAT gene. The relative ratio of CAT to a single gene, caldesmon,20 in dot blots was used as a reference for comparison of transgene copy numbers. Transgenic and nontransgenic litters were weaned at 4 to 6 weeks, and euthanized, and tissues were homogenized in buffer A and analyzed for CAT activities.18 Total proteins in various tissues were determined by the Bradford method.21 Mann-Whitney tests22 were performed for data comparisons between transgenic and nontransgenic litters of the same founders and for data comparisons between the wild-type and various mutant promoters.

To generate the cTnT-LacZ transgenic mice, plasmid BCAT-LacZ was constructed by ligation of the XhoI (fill in)–BamHI fragment from pBCAT2 with the XhoI (fill in)–BamHI fragment from pSD-KlacZPA (a generous gift of Dr Ju Chen, University of California, La Jolla, Calif) containing the LacZ and simian virus 40 poly(A) cassette. Whole-mouse β-galactosidase (β-gal) staining of cTnT-LacZ embryos was performed, and embryos were then sectioned at 7 μm.

Cell Culture

Neonatal cardiomyocytes and cardiac fibroblasts were prepared from 3-day-old rats.18 Mouse fibroblast line C3H 10T1/2 cells were grown in DMEM containing 10% FCS.

Cell Transfection and CAT and Luciferase Assays

Cells grown on a 60-mm dish were transfected by the DEAB (2,3-Dioleoyloxy)propyl(1,3)-N,N,N-trimethylammonium methylsulfate (DOTAP) method (Boehringer Mannheim) with 5 μg of test plasmid and 1 μg of reference plasmid pCH110 (Pharmacia) containing the LacZ gene. At 36 to 48 hours after transfection, CAT activities were determined18 and normalized against β-gal activities measured by the Miller23 method.

To replace the CAT reporter gene with a firefly luciferase gene, pcTNT-249 and pcTNT-20818 were digested to release the CAT cassette and ligated with the NheI–BamHI (fill in) fragment from a pG3 plasmid (Promega) containing the luciferase cassette. Cells grown on a 35-mm dish were transfected with 2 μg of cTnT promoter–firefly luciferase constructs, 0.25 μg of pCDNA1.1-MEF2C (a generous gift of Dr E.N. Olson, UT Southwestern Medical Center, Dallas, Tex), and 0.2 μg of reference plasmid pRL-TK (Promega) containing the Renilla luciferase gene. The activities of firefly and Renilla luciferase were measured by the Dual-Luciferase Reporter Assay (Promega) at 36 to 48 hours after transfection. The firefly luciferase activities were normalized against the Renilla luciferase activities.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

We have previously shown that the cTnT proximal promoter can drive a high level of reporter gene expression in cultured cardiomyocytes, but not in fibroblasts.18 This promoter contains a Nkx2.5 binding site, 2 modules (D and F with sequence homology), 2 CAUrG boxes, a MCAT motif, an AP2

(Concert). The resulting mutant promoters are called Mut1 for the 3-bp change in the core sequence of the MEF2-like motif and Mut5 for the 4-bp change in the MEF2-like motif and 1-bp change in the upstream TCTG(G/C) direct repeat. During the isolation of Mut5, we obtained a second site mutation, MutD, which includes a 13-bp deletion (from −322 to −310 bp) and a 6-bp change (gCTGcatac [changes in lowercase]) in module D. As a result, a double mutant promoter in MutD and Mut5 was generated and named Mut(D+)5.
motif, 2 plasmids, pcTNT-249-luc and pcTNT-208-luc, were constructed. The pcTNT-249-luc and pcTNT-208-luc have the cTnT promoter deleted to −249 and −208, respectively, so that pcTNT-249-luc contains the MEF2-like motif but pcTNT-208-luc does not (Figure 3A). The pcTNT-249-luc reporter construct drove high levels of luciferase expression in cardiomyocytes (22.19±3.21) but not in fibroblasts (2.34±0.21), whereas in the pcTNT-208-luc–transfected cells, reporter gene expression was at background levels in both cardiomyocytes (3.86±1.76) and fibroblasts (1.57±0.29, Figure 3B). These results were very similar to those obtained previously using CAT as a reporter gene.18 To test the effect of MEF2C on cTnT promoter activity, constructs pcTNT-249-luc and pcTNT-208-luc were cotransfected with the MEF2C expression vector (pCDNA1.1-MEF2C) or the empty control vector (pCDNA1.1) into cardiomyocytes and fibroblasts expressing as relative luciferase activities after normalization with Renilla luciferase activities. Expression of the MEF2C protein did not significantly transactivate either promoter construct in fibroblasts, whereas the expression of MEF2C alone can transactivate luciferase activity driven by a muscle-specific gene, mXin.26

Modules D and F Have an Additive Stimulatory Effect on the Expression of the cTnT Gene in Cardiomyocytes

To investigate the relative roles of module D and F within the −497-bp promoter in determining cardiac-specific expression of the cTnT gene, site-directed mutagenesis was performed and subsequent effects on the transcription of a reporter CAT gene were examined. These constructs (Figure 4A) were transfected into cultured neonatal rat cardiomyocytes and cardiac fibroblasts. The wild-type construct, BCAT2, led to high levels of CAT gene expression in cardiomyocytes (Figure 4B) but not in fibroblasts (Figure 4C). CAT expression was decreased to 69% by a 3-bp change in the core sequence of the MEF2-like motif in the F module (Mut1) and further decreased to 51% by a 5-bp mutation in the MEF2-like site and its flanking TCTGG sequence (Mut5, Figure 4B). The alteration in the D region alone (MutD) decreased CAT activity to 61% of that observed with wild-type BCAT2 (Figure 4B). Interestingly, the double mutant promoter, Mut(D+5), reduced reporter expression to 19% of that observed with BCAT2 (Figure 4B). These results suggest that the D and F modules have an additive stimulatory effect on the expression of the cTnT gene in cardiomyocytes. It is noteworthy that CAT activities of Mut5, MutD, and Mut(D+5) promoters in cardiac fibroblasts was significantly higher than that of the wild-type promoter (P<0.05, Figure 4C). These results suggest that these mutant promoters have relaxed their cardiac specificity. However, this possibility should be confirmed in transgenic mice carrying these mutant promoters. On the contrary, CAT activity of Mut1 in fibroblasts seemed to be lower than that of the wild type (Figure 4C). We confirmed these data with a luciferase reporter gene, of which the product can be measured in an easier and more sensitive way. Similar results were obtained (Figure 4D and 4E). Consistently, the luciferase activity of Mut1 in fibroblasts was significantly lower than that of the wild type (P<0.05) (Figure 4E).

The Proximal −497-bp Promoter of the cTnT Gene Can Drive Cardiac-Specific Expression In Vivo

To determine whether the proximal promoter of the cTnT gene is sufficient to drive cardiac-specific expression in vivo, transgenic mouse lines were generated using the BCAT2 construct with a LacZ or CAT reporter gene. As can be seen in Figure 5, the LacZ reporter gene driven by the −497-bp promoter is specifically expressed in the paired cardiogenic mesoderm of a day 7.5 postcoitum (p.c.) embryo (Figure 5A).
and in the looping heart tube of a day 8.0 p.c. embryo (Figure 5C). A cross section of the day 8.0 p.c. transgenic embryo showed that the \( \text{LacZ} \) gene is specifically expressed in the myocardial cells (Figure 5E). As development proceeds, \( \text{LacZ} \) expression is distributed uniformly throughout both atria and ventricles of developing mouse embryos. By day 10.5 p.c., a low level of \( \text{LacZ} \) expression starts to be detected in developing somites (data not shown). At fetal stages, \( \text{LacZ} \) expression is also detected in developing skeletal muscles, such as intercostal muscles; brachial muscles; muscles in metacarpal bones, metatarsal bones, and lower legs (compare Figure 6A and 6B); and muscles surrounding the developing vertebrae (data not shown). However, the level of transgene expression in the developing skeletal muscle is much lower than that seen in the developing heart (compare Figure 6A and 6C). In the adult, \( \text{LacZ} \) is expressed only in the heart, but not in other tissues tested, including skeletal muscles, liver, spleen, lung, kidney, brain, stomach, and intestine (data not shown). This transgene expression pattern was observed in developing mice of 3 independent founders and is very similar to that of endogenous rat \( cTnT \) gene. For BCAT2 transgenic mice with a \( \text{CAT} \) reporter gene, 4 independent transgenic founders were established. Protein extracts from various tissues of transgenic mice at 4 to 6 weeks of age were
isolated and assayed for CAT activity. Tissues from nontransgenic mice were also harvested and analyzed as negative controls. In all BCAT2 transgenic lines, CAT is strongly expressed in the heart but not in other tissues, including intestine, lung, liver, kidney, spleen, skeletal muscle, stomach, and brain (see Table I online, http://www.circresaha.org). These results suggest that the proximal −497-bp promoter of the cTnT gene is sufficient to confer cardiac-specific expression in vivo and faithfully recapitulates endogenous cTnT expression during development.

Modules D and F Have Additive Stimulatory Effects in the Heart and Inhibitory Effects in the Stomach

To understand the roles of the D and F modules in regulating cardiac-specific expression in vivo, constructs carrying Mut1, Mut5, MutD, or the Mut(D+5) mutation were used to generate transgenic mouse lines. Except line 5228/2, the relative copy number of transgene in all founder lines used in this study ranged from 1 to 7, as normalized with a single-copy caldesmon gene. However, there was no obvious correlation between copy numbers and expression levels of the transgene in these lines. In transgenic mice carrying a 3-bp change in the A/T-rich core of the MEF2-like motif (Mut1), CAT activity was slightly decreased (P=0.12) in the heart as compared with wild-type BCAT2 transgenic lines and was undetectable in other tissues (see Tables II and VI online, http://www.circresaha.org). In transgenic mice carrying the construct with a 4-bp change in the MEF2-like motif and a 1-bp change in the TCTGG sequence (Mut5), CAT expression was further decreased in the heart as compared with BCAT2 (P=0.06) and as compared with Mut1 (P<0.05, see Tables III and VI online, http://www.circresaha.org; Figure 7). Interestingly, CAT activity was also detected in the stomach (P<0.05) of transgenic mice of all analyzed founders carrying the Mut5 construct (see Table III online, http://www.circresaha.org; Figure 7) and in the intestine of line 5185/2 and 5228/2 transgenic mice (see Table III online http://www.circresaha.org). These results suggest that the A/T-rich core of the MEF2-like motif and its flanking sequence have dual effects on the cTnT expression—a stimulatory effect in the heart but an inhibitory effect in the stomach. In transgenic mice carrying mutations disrupting the upstream TCTG(G/C) direct repeat (MutD), CAT expression remained cardiac specific (P<0.05, see Table IV online, http://www.circresaha.org), but the expression level was dramatically decreased (P<0.05, see Table VI online, http://www.circresaha.org; Figure 7). In transgenic mice carrying double mutations in both the D and F modules (Mut[D+5]), CAT activity in the heart was greatly diminished (see Table V online, http://www.circresaha.org), whereas CAT expression in the stomach and intestine were increased (P<0.05) as compared with the BCAT2 transgenic mice (see Tables V and VI online, http://www.circresaha.org; Figure 7). In transgenic line 2698/1 and 2707/2, CAT expression was also detected in the brain (P<0.05, see Table V online, http://www.circresaha.org). These results confirm that the D and F elements have additive functions in controlling cTnT expression.

Discussion

In the present study, we have shown that in transgenic mice, the proximal −497-bp promoter of the rat cTnT gene is able
to drive cardiac-specific expression of a reporter gene since early embryonic stages. The temporal and spatial expression patterns of transgenes driven by the cTnT proximal promoter are different from that driven by α-myosin heavy chain (MHC) and β-MHC or ventricular myosin light chain 2 promoters, which are commonly used in transgenic overexpression experiments. The ventricular myosin light chain 2 promoter is used to drive ventricle-specific expression. The β-MHC promoter can drive reporter gene expression in the developing heart; however, the expression level decreases dramatically after birth. On the other hand, the α-MHC promoter is often used to drive gene expression after birth, although this promoter is very active in the atria of embryonic heart. Different from these promoters, the −497-bp cTnT promoter drives reporter expression in myocardial cell stages throughout both atria and ventricles at all developmental stages. Although the reporter is also transiently expressed in the developing skeletal muscles at fetal stages, the expression level is much lower than that in the heart. Thus, the −497-bp cTnT promoter may serve as an alternative to drive the expression of a foreign gene in both developing and adult mouse hearts. In addition, the present study identified 2 critical elements within the −497-bp cTnT promoter, the D and F modules, which act together to achieve a high level of expression specifically in the heart. Sequence analysis reveals that a portion (−319 to −289 bp) of the D module has a high degree of sequence homology to the F module. Similar to the F module, the D module contains an A/T-rich site and 3 TCTG(G/C) sequences potentially forming 2 direct repeats but no MEF2-like motif. Mutations that disrupt these direct repeat sequences in the D module (MutD) greatly diminish promoter activity in the heart but do not alter the cardiac specificity of transgene expression (see Table IV online, http://www.circresaha.org). This result suggests that the D module may function as an enhancer to increase transcription of the cTnT gene. In a separate study using cultured cardiomyocytes, we have shown that a synthetic D2 module containing a TCTG(G/C) direct repeat and an A/T-rich site to the F module (MutD) greatly diminish promoter activity in the heart but do not alter the cardiac specificity of transgene expression (see Table III online, http://www.circresaha.org). Coincidentally, one of the 5-bp changes in this Mut5 promoter is located in the TCTG(G/C) direct repeat of the F module, which contains the F module but lacks the sequences from −250 to −497 bp. As such, the resulting activity is similar to that of the −497-bp promoter (Wang Q and Lin JJ-C, unpublished data, 1999).

Mice transgenic for a promoter containing mutations within the F module (Mut5) exhibit lower transgene expression and less cardiac specificity (see Table III online, http://www.circresaha.org). Coincidentally, one of the 5-bp changes in this Mut5 promoter is located in the TCTG(G/C) direct repeat of the F module, suggesting that this novel repeat sequence may play an important role in controlling cardiac-specific expression of the cTnT gene. This idea is further supported by our recent studies on this direct repeat element. In transfection studies on cultured cardiomyocytes, single-bp mutations in the TCTG(G/C) direct repeat of the F module, which either destroys the repeat sequence or creates a perfect repeat, resulted in an alteration in binding affinity and a significant decrease in −249-bp cTnT promoter activity (Wang Q and Lin JJ-C, unpublished data, 1999). Double mutations in both D and F modules (Mut(D+5)) actually altered both upstream and downstream TCTG(G/C) direct repeats. As a result of these alterations, an additive effect on reporter gene expression was observed in Mut(D+5) transgenic mice.

Given the importance of MEF2 factors in the regulation of other cardiac-specific genes, the MEF2-like sequence located in between the direct repeat and the A/T-rich site of the F module may be critical for cTnT promoter activity. However, in this study, we failed to demonstrate that MEF2A, MEF2C, or HF1b factor is capable of recognizing the MEF2-like sequence and that overexpression of MEF2C can transactivate the cTnT promoter in cardiomyocytes or fibroblasts. Moreover, we found that a 3-bp mutation within the A/T-core sequence of the MEF2-like motif (Mut1) decreased the promoter activity in both cardiomyocytes and fibroblasts (Figure 4D and 4E), suggesting that the function of this A/T sequence in the cTnT promoter may be ubiquitous rather than cardiac specific. Taken together, these data suggest that the MEF2-like motif in cTnT promoter may act as another A/T-rich site.

There is an A/T-rich sequence found in both D and F modules. Interestingly, a GATA binding site is located in the antisense strands of each these A/T-rich regions. Furthermore, there is another GATA site found in the antisense strand of the TCTG(G/C) direct repeat within the F module, and a reverse GATA site is located within the MEF2-like motif. Presently, the role of these A/T-rich/GATA sites in regulating the expression of the cTnT gene remains unknown. Our preliminary studies revealed that cotransfection of a GATA-4 expression vector and a plasmid containing the cTnT promoter–luciferase fusion gene into nonmuscle cells did not show significant transactivation. However, there is a Nkx2.5 consensus sequence found in the rat cTnT promoter from −381 to −375 bp. Recently, it has been shown that Nkx2.5 and GATA-4/5 can directly interact and synergistically transactivate certain cardiac muscle genes.27–29 Therefore, these A/T-rich/GATA sites and the Nkx2.5 site found in regulating the expression of the cTnT gene remains unknown. Our preliminary studies revealed that cotransfection of a GATA-4 expression vector and a plasmid containing the cTnT promoter–luciferase fusion gene into nonmuscle cells did not show significant transactivation. However, there is a Nkx2.5 consensus sequence found in the rat cTnT promoter from −381 to −375 bp. Recently, it has been shown that Nkx2.5 and GATA-4/5 can directly interact and synergistically transactivate certain cardiac muscle genes.27–29 Therefore, these A/T-rich/GATA sites and the Nkx2.5 site found in regulating the expression of the cTnT gene remains unknown.
factor is not involved in the regulation of cTnT gene expression.

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References

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