Published in final edited form as:

Anat Rec (Hoboken). 2008 December ; 291(12): 1574–1586. doi:10.1002/ar.20772.

Requirement of TCTG(G/C) Direct Repeats and Overlapping GATA Site for Maintaining the Cardiac-specific Expression of *Cardiac Troponin T* in Developing and Adult Mice

Shannon M. Harlan[#], Rebecca S. Reiter[#], Curt D. Sigmund^{*}, Jenny Li-Chun Lin[#], and Jim Jung-Ching Lin[#]

[#] Department of Biology, University of Iowa, Iowa City, IA, 52242-1324

^{*} Department of Internal Medicine, University of Iowa, Iowa City, IA, 52242-1324

Abstract

The cardiac-specific −497bp promoter of rat *cardiac troponin T* (*cTnT*) contains two similar modules, D and F, each of which possesses TCTG(G/C) direct repeats and A/T-rich sites. To identify cis-elements critical for cardiac specificity, a −249bp promoter containing only module F and its site-directed mutations were used to generate transgenic mice. Transgene expression of the −249bp promoter remained cardiac-specific, despite low and non-uniform expression. The non-uniform expression pattern of the transgene coincided with differential expression of HMGB1, which appeared to be the predominant form of HMGB family proteins in the heart. The HMGB1 binds to the A/T-rich/MEF2-like sites of the *cTnT* promoter, as determined by chromatin immunoprecipitation assays. Mice carrying the −249bp promoter with point mutations disrupting the direct repeats expressed transgene at lower levels in the heart and ectopically in the brain. Ectopic expression of transgene was also observed in developing limbs and head. These results suggest an important role for the direct repeat in determining the cardiac specificity. Furthermore, mice carrying a mutant promoter simultaneously disrupting the direct repeats and overlapping GATA site failed to express the transgene in any tissues tested. Therefore, the direct repeat and overlapping GATA site are critical for the expression level and cardiac specificity. The F module controls one level of cardiac specificity. For a uniform and high level of cardiac-specific expression, the upstream element (−497bp to −250bp) is further required, possibly through the D enhancer module and the combination of Nkx2.5 and GATA sites.

Keywords

A/T-rich site; MEF2-like motif; HMGB1; ChIP; cis-regulatory element

Introduction

Cardiac troponin T (*cTnT*) gene expression is detected in the heart starting at approximately 7.5 days post conception (dpc), in the somites starting at approximately 10.5dpc, and transiently in the skeletal muscle of the developing mouse. However, expression is restricted in the adult heart (Wang et al., 2001). This expression pattern suggests that *cTnT* transcription is regulated differently during development than in the adult. Previous promoter analysis identified the −497bp proximal promoter of the rat *cTnT* gene to be

sufficient to drive a *LacZ* reporter in an expression pattern identical to that of endogenous *cTnT* expression throughout development and in the adult (Wang et al., 1994; Wang et al., 2000; Wang et al., 2002). Recently, a transgenic *cTnT-Cre* mouse line, in which transcription of Cre recombinase was driven by the -497bp promoter, was generated and successfully used in combination with a Cre/loxP system to specifically inactivate *Bmp4* (Jiao et al., 2003), *Smad4* (Song et al., 2007), and *Fgf8* (Ilagan et al., 2006) in early cardiomyocyte lineages starting from 7.5dpc. The -497bp promoter contains two similar modules, termed module D and module F, each containing at least one pair of TCTG(G/C) direct repeats and an A/T-rich site. The F module is unique as it also contains a MEF2-like motif and a GATA consensus sequence overlapping with the direct repeats (Fig. 1). Previously, it was shown that the F module can confer cardiac specificity in cultured cardiomyocytes and fibroblasts, whereas the D module may function as an enhancer (Wang et al., 1994; Wang et al., 2000; Wang et al., 2002). Using gel mobility shift assays, DNA footprint analysis, and Southwestern cloning, we further identified cardiac-specific 42kDa protein(s) bound to the direct repeats and cloned a protein of the HMGB family of proteins bound to the A/T-rich sites (Wang et al., 2002). Factors other than the 42kDa protein(s) were shown to bind the direct repeats in non-cardiac tissues (Wang et al., 2002), suggesting that the direct repeats both positively and negatively regulate *cTnT* expression.

We used a transgenic mouse approach to determine whether the F module alone drives cardiac-specific gene expression, and what roles do the direct repeat and its overlapping GATA site of the F module have in driving cardiac-specific gene expression. The first construct (249LacZ) containing -249bp promoter fused to *LacZ* (Fig. 1) was designed to identify if the F module alone was sufficient to drive cardiac-specific expression. To identify the role of the direct repeats within the -249bp promoter, a second construct (F2DLacZ) containing a 2 base pair mutation to disrupt the direct repeats was used to drive *LacZ* expression. To examine the role of the direct repeats and flanking sequence, a third construct (FCaLacZ) containing a point mutation in the -249bp promoter to disrupt both the direct repeat and overlapping GATA site was generated. Using these three transgenic founders, we identified that the F module alone can confer cardiac specificity, however, the transgene is non-uniformly expressed in the heart. To understand the mechanisms underlying the non-uniform expression, we used chromatin immunoprecipitation (ChIP) assays to show the *in vivo* binding of HMGB1 on the A/T-rich/MEF2-like site of the *cTnT* promoter and immunohistochemical stains on serial sections of transgenic heart to show similar expression patterns of the HMGB1 with the transgene expression. These results suggest that HMGB1 may be a key factor in the regulation of the -249bp *cTnT* promoter.

Materials and Methods

Western blot analysis

Total protein extracts were prepared from mouse heart tissues and subsequently analyzed by Western blot analysis as previously described (Warren et al., 1994b; Sinn et al., 2002). The primary antibodies used include rabbit anti-HMGB1/2 (which we will refer to as anti-HMGB1) antibody at 1:200 dilution (ab23745), rabbit anti-HMGB2 antibody at 1:50 dilution (ab11973) (Abcam, Inc., Cambridge, MA), and monoclonal anti-GAPDH antibody at 1:2000 dilution (Research Diagnostics, Inc., Flanders, NJ). The antigens used to generate rabbit polyclonal antibodies are synthetic peptide "DAAKKGVVKAEEKSK" of HMGB1 for the anti-HMGB1/2 antibody and synthetic peptide "KSEAGKKGPGRPTGSK" of HMGB2 for the anti-HMGB2 antibody (Abcam, Inc.). Because the anti-HMGB1/2 antibody was demonstrated in this study to be specific to HMGB1 but not HMGB2, we will refer here to the anti-HMGB1/2 as anti-HMGB1. For quantitation, Western blot results in various exposures from 3 independent sample sets were scanned with Labworks software (UVP Bioimaging Systems, Upland, CA) to quantify relative band intensities for HMGB1 and

GAPDH. The GAPDH level in each sample was used as an internal loading control. The differences in relative ratios of HMGB1 to GAPDH for atria and ventricles were statistically analyzed by t-test.

Plasmid construction and generation of transgenic mice

Various *cTnT* promoter-LacZ plasmids were generated by subcloning the *cTnT* promoter XhoI/BamHI fragment from previously constructed plasmids, cTNT-249-luc, F2D-luc, and FCa-luc (Wang et al., 2002) into the XhoI/BamHI-digested pSD-KlacZPA vector (a generous gift of Dr. Ju Chen, University of California San Diego, La Jolla, CA). The resulting plasmids, pcTNT-249-LacZ, pF2D-LacZ, and pFCa-LacZ, were used to isolate BamHI/NotI promoter-LacZ fragments for generating transgenic mice as previously described (Sigmund, 1993). Procedures were all carried out at the transgenic core facility at the University of Iowa. Mice positive for the transgene were identified by PCR genotyping of tail DNA as previously described (Wang et al., 2000). The previously generated transgenic line, 497LacZ, containing *LacZ* reporter gene driven by the -497bp *cTnT* promoter (Wang et al., 2000) was used as a control for comparison of expression pattern. All transgenic lines studied here did not exhibit any phenotypes as compared to non-transgenic littermates.

Analysis of transgenic mice

LacZ expression was determined by X-gal staining as previously described (Wang et al., 2000; Wang et al., 2001) using whole embryos or dissected adult tissues. After whole-mount staining, selected embryos were dehydrated and embedded in Paraplast Plus paraffin under vacuum pressure. Embryos were then sectioned on a microtome at 7 μ m, and counter-stained with hematoxylin and eosin (Wang et al., 1996). For frozen section analysis, the heart was dissected out, washed in ice cold PBS, mounted in OCT, and flash frozen in liquid nitrogen-cooled isopentane at -160°C (Yang et al., 1998). Sections were prepared at 10 μ m on a cryostat and collected on Superfrost Plus slides. After air dry, sections were formalin fixed for 10 minutes at 4°C, washed in PBS, stained overnight with X-gal staining solution, followed by counter staining with nuclear fast red.

Dot blot hybridization

Genomic DNA was isolated from the liver of transgenic mice. Three fold serial dilutions were made from 5.4 μ g/ml of DNA sample diluted in 0.4M NaOH and 10mM EDTA. DNA was denatured for 10 minutes at 100°C and kept on ice. A pre-wetted Zeta-probe GT membrane was placed on the Biodot microfiltration apparatus and 0.5ml of DNA sample was loaded in each well (BioRad Laboratories, Hercules, CA). After wash in 0.4M NaOH, the blot was cross linked using UV light at energy setting 1200 on UVC-515 ultraviolet multilinker (Ultra-Lum, Inc., Claremont, CA). A 900bp XbaI *Caldesmon* cDNA fragment from pCBCaD39 (Warren et al., 1994a) and a 2kb EcoRV/BamHI *LacZ* fragment from pBCAT-LacZ (Wang et al., 2000) were labeled with α -P³²-dCTP (Amersham Biosciences, Piscataway, NJ) using the random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed using 1 $\times$ 10⁶cpm/ml of probe, at 68°C for the *LacZ* probe and at 50°C for the *CaD39* probe for 2 hours in QuikHyb solution (Stratagene, La Jolla, CA) (Wang et al., 2000). The membranes were washed 2 times in 1XSSC, 0.1% SDS at hybridization temperature, followed by 1–3 washes in 0.1XSSC, 0.1% SDS. The membranes were exposed to X-ray film using intensifying screen at -70°C. The intensities of the dots were quantified using Labworks software. Relative transgene copy was determined by normalizing the *LacZ* probe to the *CaD39* probe for DNA loading control.

Immunohistochemistry

Serial frozen sections of adult hearts were prepared as described above, with alternative sections used for X-gal staining or immunohistochemistry. For immunohistochemistry, sections were mounted on superfrost Plus slides and processed following the protocol provided by the Vectastain ABC kit (Vector Laboratories, Burlingame, MA). Briefly, sections on the slides were fixed in 10% buffered formaldehyde for 5 minutes, followed by a quick rinse in PBS. Sections were then treated in cold acetone for 3 minutes, 0.3% hydrogen peroxide for 10 minutes, rinsed in PBS, followed by immunostaining with anti-HMGB1 antibody at 1:500 dilutions in PBS. Antibody detection was performed using diaminobenzidine (DAB) according to the manufacturer's instructions. Sections to be used for X-gal staining were processed as described above.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described with a few modifications (Turck et al., 2004). Mouse hearts were dissected, washed, minced into small pieces and cross-linked in 1% formaldehyde for 15 minutes. After quenching in 0.1M glycine solution, the heart tissues were pulverized to powder in liquid N₂ and then suspended in 70% glycerol buffer (20 mM Hepes, pH7.4, 5 mM MgCl₂, 5 mM KCl, 50 mM saccharose, 70% glycerol and 1 mM DTT), from which the nuclei were isolated as previously described, except the omission of the nylon filtration step (Turck et al., 2004). Formaldehyde cross-linked nuclei were resuspended in sonication buffer (10 mM Hepes, pH7.4, 1 mM EDTA and 0.25% SDS) and sonicated 12 times, for 15 seconds each at 45% setting (Vibra Cell High Intensity Ultrasonic Processor, Sonics & Materials, Inc, Danbury, Ct) to shear chromatin to an average size of 1.5kb. The sonicated chromatin (~50–100 µg) was incubated with 2µg BSA and 30µl of prewashed protein A/G agarose beads (Pierce, Rockford, IL) for 1 hour at 4°C and then centrifuged at 12,000xg for 15 minutes. The pre-cleared chromatin was further incubated with 10µl of anti-HMGB1 for 4 hours at 4°C. Then 30µl of prewashed protein A/G agarose beads were added, and incubated overnight at 4°C with gentle shaking. The beads containing immunoprecipitates of protein/DNA complexes were collected and washed as described (Turck et al., 2004). The protein/DNA (immunoprecipitates) complexes were eluted from beads as described (Turck et al., 2004), further treated with 50µg/ml RNase A for 30 minutes at 37°C, followed by overnight incubation with 10µg proteinase K at 37°C, and 8 hour incubation at 65°C. The DNA was then recovered from the treated immunoprecipitates by standard phenol/chloroform extraction and ethanol precipitation and used in PCR reactions using the primer pair, c (5' GCGTGGGGTACTTGTTCCTTTAGC 3') and d (5' CGCCATTCGCCATTCAG 3'), which is immediate downstream of the A/T-rich/MEF2-like sites and flanking both the *cTnT* promoter and LacZ transgene. To identify if HMGB1 could bind endogenous *cTnT* promoter, the PCR reactions used the primer pair, a (5' GGCGAGCCAGACCAACAGC 3') and b (5' ACGCCAGAAGCCCAGGTCCAG 3'), which is immediately upstream of the A/T-rich/MEF-like sites of the endogenous *cTnT* promoter. Negative control primer pair flanking two MEF2 sites of the *mXina* promoter included primer e (5' GGGCTTTGGGCTCGGTGGAC 3') and primer f (5' GGGCGGGGGAGAGGCTATGTT 3') (Lin et al., 2001). Input DNA was used in the same PCR reactions as a positive control.

Results

The F module alone confers cardiac specificity in the 249LacZ transgenic mice

To examine whether the F module alone can drive cardiac-specific expression in vivo, the 249LacZ transgenic lines carrying –249bp proximal promoter driving *LacZ* gene were generated. Three independent founders were obtained: 249LacZ-1, 249LacZ-2, and 249LacZ-3. In these founders transgene expression was detected in both the atria and

ventricles in a non-uniform, variegated pattern with more pronounced expression on the dorsal-right side as compared to the ventral-left side (Fig. 2). Images are representative staining patterns from analyzing at least 3 independent hearts of each founder from the 249LacZ transgenic mice. These results are in stark contrast to the 497LacZ transgenic founder carrying the -497bp proximal promoter driving the *LacZ* gene. In the 497LacZ mice, transgene expression was consistently detected homogeneously throughout the adult heart (Fig. 2A and B). Transgene expression was not detected in other tissues tested such as brain, spleen, liver, lung, skeletal muscle, dorsal aorta, and small intestine (data not shown except brain shown in Fig. 7A and B). Therefore, the transgene expression driven by the -249bp proximal promoter containing only the F module remained cardiac-specific, although it did not completely mimic the endogenous *cTnT* expression pattern in the adult heart.

Transgene expression patterns among the three 249LacZ founders were very similar, despite the difference in the expression levels. The expression levels appeared to correlate well with the relative copy number of the transgene as quantified by Southern hybridization, with the high expressing 249LacZ-1 founder having approximately the same relative copy number as our 497LacZ positive control (Supplemental Fig. 1). The relative copy number was calculated by reprobing the blot for a single copy gene, *Caldesmon* and normalizing the hybridization intensity of each dot with its respective intensity obtained by this caldesmon probe. Relative copy numbers were as follows: 249LacZ-1 copy number 7, 249LacZ-2 copy number 5, 249LacZ-3 copy number 3, and the 497LacZ positive control having a relative copy number of 7.

To thoroughly characterize the expression pattern of the 249LacZ transgenic mice, frozen serial sections were prepared from adult hearts and stained with X-gal. Expression profiles were similar in all three founders. Transgene expression was consistently prominent in the interventricular septum (IVS), atria, and anterior region of the ventricles (Fig. 3B, C, and D). Weaker expression was detected in the posterior region of the left and right ventricles. In addition, expression in the right atrium was consistently stronger than in the left atrium. These staining patterns are in contrast to the expression pattern observed in the 497LacZ founder, where transgene expression was seen homogeneously throughout the atria and ventricles of the adult heart (Fig. 3A). X-gal staining was not detected in non-transgenic littermates (Fig. 3E).

The 249LacZ transgenic mice can mimic endogenous *cTnT* expression in development

To identify the role of the F module in *cTnT* expression during development, the 249LacZ transgenic mice were examined for transgene expression throughout embryogenesis. Similar to the 497LacZ line (Fig. 4A), the 249LacZ founders all expressed the transgene in the heart forming fields of the embryos at 7.5dpc (Fig. 4E). Transgene expression was detected throughout the myocardium of the developing heart at the linear heart tube stage (8.5dpc) in both the 497LacZ and 249LacZ transgenic mice (Fig. 4B and F). However, after cardiac looping and the morphological demarcation of the four cardiac chambers emerge at 10.5dpc, transgene expression of the 249LacZ mice becomes non-uniform in the developing heart, yet remained cardiac-specific (Fig. 4G). On the other hand, transgene expression in the 497LacZ line (Wang et al., 2000) is uniformly distributed throughout the heart at this stage (Fig. 4C). Similar expression patterns in developing somite and skeletal muscle were detected in both the 497LacZ and 249LacZ-1 transgenic embryos at 12.5dpc (Fig. 4D and H) but not in 249LacZ-2 and 249LacZ-3 embryos. Using paraffin embedded, H&E-stained sections of whole-mount X-gal stained embryos to verify the expressed tissues as developing somite and muscle, we again detected transgene expression only in 497LacZ line (Fig. 5A and B) and 249LacZ-1 founder (Fig. 5C and D) but not in the two low expressing founders (249LacZ-2 and 249LacZ-3) (Fig. 5E and F).

The TCTG(G/C) direct repeats are critical in maintaining cardiac specificity in development and in the adult

To investigate the role of TCTG(G/C) direct repeat in the cardiac-specific expression of the *cTnT* gene, we generated transgenic mice (F2DLacZ) carrying transgene driven by a 2-bp mutation (F2D in Fig. 1) of -249bp promoter, which destroys the TCTG(G/C) direct repeat but leaves the overlapped upstream GATA site intact. Eight founders were obtained using the F2DLacZ construct, which were put into two categories based on their expression levels. Again, expression levels correlated with relative copy numbers as determined by Southern dot blot hybridization, with our high expressing founders having a relative copy number of 7 and the low expressing founders having a relative copy number of 1 (data not shown). Overall, cardiac expression levels were much less than that of the 249LacZ lines with the same copy number of transgene. The low expressing category, F2DLacZ-A, consisted of four founders all of which expressed the transgene at detectable levels in the atria with very low expression in the ventricles (Fig. 6A and B). The high expressing category, F2DLacZ-AV, also consisted of four founders. The F2DLacZ-AV founders all exhibited relatively strong, non-uniform expression in the atria, as well as non-uniform expression in the ventricles (F2DLacZ-AV) (Fig. 6D and E). Using serial sections stained with X-gal, expression was consistently detected in the atria for the F2DLacZ-A line, with expression in the ventricles being restricted to the IVS at very low levels (Fig. 6C). Serial sections of the F2DLacZ-AV hearts revealed expression in the atria, IVS, and right ventricle (Fig. 6F). Expression was not detected in the left ventricle (Fig. 6F). Staining in the right ventricle was typically observed to be in the anterior region, as seen in 249LacZ line (Fig. 3B and C).

In addition to a significant reduction in transgene expression levels in F2DLacZ adult hearts, ectopic expression was observed in the adult brain of the F2DLacZ-AV mice. Transgene expression was detected in the olfactory lobes, cerebrum, midbrain and cerebellum but not in the medulla oblongata (Fig. 7C and D), although the level of ectopic expression varied among all founders. In addition, transgene expression was found in the vessels of the brain. LacZ staining was not detected in the brains of non-transgenic littermates (data not shown) or the 249LacZ mice (Fig. 7A and B). Transgene expression was not detected in any other adult tissues tested such as lung, kidney, skeletal muscle, or liver (data not shown).

Ectopic expression during development was also observed in the F2DLacZ-AV founders but not in the F2DLacZ-A founders. The F2DLacZ-A founders at 11.5dpc expressed the transgene in the developing heart and somites (Fig. 8A and B), similar to endogenous *cTnT*. In contrast, the high expressing F2DLacZ-AV founders at this stage displayed ectopic expression of the transgene (Fig. 8C and D). Transgene expression was detected in the developing limb buds as early as 11.5dpc, when *cTnT* expression is not normally expressed in this tissue (Fig. 8C and D). The range of ectopic expression varies from mild to more severe cases in which expression was detected in the developing heart, somites, skeletal muscle, spinal chord, head, and mesenchymal condensation of the developing limbs and surrounding the spinal chord (Fig. 8D). Expression in the developing heart was also reduced, with expression predominantly seen in the developing atria (Fig. 8D).

A single bp change (FCa) in -249bp promoter with the disruption of direct repeat and its overlapping GATA site completely abolishes the transgene expression

Among 5 founders that were generated with FCaLacZ construct, none of them showed any detectable expression of transgene in adult tissues and developing embryos (data not shown). To verify that the lack of expression was not due to lower copy numbers of the transgene, dot blot hybridization was performed on all five founders (data not shown). Relative copy numbers were comparable to those obtained for our 249LacZ and F2DLacZ expressing founders, suggesting that copy number was not a factor in the lack of expression

observed for this construct. Moreover, we identified one founder as having a relative copy number of 23 as compared to relative copy number of 7 for our high expressing 249LacZ-1 founder, demonstrating that even a three fold increase in copy number failed to express the transgene in any tissues tested. This mutant promoter (FCa-luc) has been previously shown to have a very little promoter activity in driving *luc* expression in cultured cardiomyocytes, like the deletion dead promoter (cTNT-208-luc) totally lacking D and F modules (Wang et al., 2002). Therefore, we considered this FCa mutant promoter as a dead promoter.

Transgene expression pattern in adult hearts roughly parallels expression of HMGB1

In the adult hearts of transgenic mice, a transition from uniform expression to non-uniform, variegated expression pattern correlated with a loss of the cis-element between -497bp and -249bp of *cTnT* promoter. Despite this difference, both the 497LacZ and 249LacZ promoters mimic the cardiac-specific expression profile of endogenous *cTnT* in developing embryos and adult mice. Furthermore, we have previously shown that over-expression of HMGB2 in cultured cardiomyocytes trans-activates reporter gene expression driven by the -249bp promoter, and on the other hand, over-expression of HMGB2 in cultured nonmuscle cells is capable of repressing reporter gene expression (Wang et al., 2002). Therefore, it is possible that the variegated transgene expression patterns observed in the 249LacZ mice may be due to the differential expression of HMGB proteins in the heart.

HMGB family of proteins is known to contain two highly homologous and interchangeable HMGB1 and HMGB2 proteins (Zappavigna et al., 1996; Boonyaratanakornkit et al., 1998; Aidinis et al., 1999; Ronfani et al., 2001). As shown in Fig. 9A, the anti-HMGB1 failed to recognize the previously described bacterially induced β -galactosidase-HMGB2 fusion protein (Wang et al., 2002). In contrast, the anti-HMGB2 antibody recognized this HMGB2 fusion protein, but surprisingly could not detect any form of HMGB family proteins from ventricular and atrial protein extracts (Fig. 9A). This result demonstrates, for the first time, that the mouse heart expressed a very little amount, if any, of HMGB2. Multiple tissue Western blot results further support this result (Fig. 9B). It is clear that the HMGB1 expression is ubiquitous including heart, skeletal muscle, brain, lung and thymus, whereas the expression of HMGB2 is restricted to lung and thymus (Fig. 9B).

To identify if a correlation between HMGB1 expression and the expression patterns of our transgenic mice existed, we examined the expression levels of HMGB1 in the atria versus the ventricles of the adult heart, as we consistently observed increased transgene expression in the atria versus the ventricles in 249LacZ and F2DLacZ transgenic mice. Using the HMGB1 specific antibody, we detected an 11-fold higher of HMGB1 protein in the atria than that in the ventricles (Fig. 10). Immunohistochemical staining using the anti-HMGB1 antibody and X-gal staining were further performed on adjacent frozen sections of 249LacZ mouse hearts. As shown in Fig. 11B, the HMGB1 staining pattern is non-uniform with more prominent staining in the right atrium, the IVS and right ventricle with weak staining in the left atrium. This pattern is very similar to the X-gal stained transgene expression pattern (Fig. 11A). Of note the sections chosen are to show maximum level of transgene expression, as well as try to show the four chambers and the IVS. The strong staining in the IVS roughly correlates with transgene expression. However, the IVS is just a small portion of the ventricle and thus overall HMGB1 staining in the ventricle is reduced when compared to the atria after normalized with GAPDH or total protein levels, which is supported by our Western blot data. These results not only provide new insight into the non-uniform expression of 249LacZ transgene, but also support the existence of a positive regulatory role for HMGB1 in regulating the *cTnT* promoter in vivo.

The HMGB1 binds to the A/T-rich/MEF2-like sites of endogenous and transgenic *cTnT* promoters but not to the MEF2 sites of endogenous *mXina* promoter

To demonstrate the *in vivo* binding of the HMGB1 proteins to the *cTnT* promoter, a chromatin immunoprecipitation assay (ChIP) was performed. Cross-linked chromatin were prepared from mouse hearts and used in ChIP assays with anti-HMGB1 antibody. Using primers pair, a/b, designed specifically around the endogenous *cTnT* promoter (Fig. 12A), we could amplify a 405bp fragment from the immunoprecipitates of 497LacZ mouse hearts by anti-HMGB1 antibody but not from the precipitates without primary antibody (Fig. 12B, lanes 1 and 4, respectively). Similar 405bp fragment was amplified from the immunoprecipitates of wild-type heart by anti-HMGB1 antibody (data not shown). Using primer pair, c/d, immediately downstream of the A/T-rich/MEF2-like sites and covering the *cTnT* and *LacZ* genes (Fig. 12A), we could amplify a specific 597bp fragment from the immunoprecipitates of 497LacZ and 249LacZ mouse hearts by anti-HMGB1 antibody (Fig. 12B, lanes 2, 3, and 6). These results suggest that the HMGB1 binds both endogenous *cTnT* gene and transgenes. There was no DNA band that could be amplified from the precipitates without anti-HMGB1 antibody (Fig. 12B, lane 4). As another control for ChIP assay, the primer pair, e/f, designed around the two MEF2 sites of the *mXina* promoter failed to produce a band from the anti-HMGB1 immunoprecipitates, however, the same primer pair was able to amplify a 322bp fragment from the input heart extracts (Fig. 12B, lanes 5 and 7). Thus, HMGB1 can bind to the A/T-rich/MEF2-like sites of the both endogenous *cTnT* and transgene promoter but not to the MEF2 sites of the *mXina* promoter *in vivo*.

Discussion

The upstream sequence from −497bp to −250bp of the *cTnT* promoter is necessary to recapitulate endogenous *cTnT* expression pattern in the heart, but is not essential for maintaining minimal cardiac specificity

In the present study, we show that the −249bp proximal promoter of rat *cTnT* gene, which contains TCTG(G/C) direct repeat, GATA consensus sequence, MEF2-like sequence, and an A/T-rich site, is sufficient to drive cardiac-specific expression of a transgene *in vivo*. Transgene expression levels in the 249LacZ transgenic mice are greatly reduced when compared to the 497LacZ mice carrying −497bp promoter. In addition, the expression pattern in the 249LacZ mouse heart was non-uniform in the atria and ventricles, which was strikingly different than the 497LacZ mice that express transgene homogeneously throughout the heart. The uniform staining seen in the 497LacZ hearts did not appear to be due to an artifact of overstaining, because the X-gal staining terminated at earlier time points (such as 60 minutes) already showed uniform expression of the transgene in the 497LacZ mouse hearts. Staining pattern was similar to those allowed to stain for 24 hours (data not shown). Thus, the upstream region −497bp to −250bp is required for uniform expression of *cTnT* gene. The −249bp promoter provides a level of cardiac specificity for certain populations of cardiomyocytes such as those in interventricular septa and atria. Overall these results demonstrate that the *cTnT* promoter is composed of different modules that control expression in different domains of the heart, which is a common phenomena observed in cardiac genes (Bodmer, 1993; Firulli and Olson, 1997; Fishman and Olson, 1997; Franco et al., 1997; Franco et al., 1998; Reecy et al., 1999; Tanaka et al., 1999; Habets et al., 2003). Therefore, it appears that all cardiomyocytes in adult hearts are not equal in terms of their transcriptional activity.

The finding further suggests that the upstream sequence from −497bp to −249bp of the *cTnT* promoter is critical not only for expression levels, but also for homogeneous expression of *cTnT* gene in the heart. Within this region, the upstream D module (−335bp to −289bp), which contains additional TCTG(G/C) direct repeat and A/T-rich sites, has

previously shown to act as an enhancer (Wang et al., 2002). The upstream Nkx2.5 binding site (−382bp to −375bp) may also participate in the high level and uniform expression of transgene in the 497LacZ mice. Previous trans-activation studies revealed that expression of Nkx2.5 alone in cultured fibroblasts could not activate reporter gene expression under the control of −497bp promoter, whereas co-expression of Nkx2.5 and GATA4 greatly enhanced the −497bp promoter activity (Lin et al., 2001). This synergistic activation has been also observed for cardiac *atrial natriuretic factor* (ANF), *brain natriuretic peptide* (BNP) and *cardiac α -actin* promoters (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998). Therefore, these two cis-regulatory elements in the −497bp promoter can play another level of control for the expression level and cardiac specificity. Nevertheless, this upstream region (−497bp to −250bp) is not essential for maintaining minimal cardiac specificity since transgene expression in 249LacZ mice remains cardiac-specific. In summary, there appears to be two levels of controls in the −497bp promoter. A combination of Nkx2.5 and GATA4 binding sites, and a collaboration of direct repeats and A/T-rich site in −497bp *cTnT* promoter regulate the expression level and cardiac specificity, and recapitulate the endogenous *cTnT* expression pattern.

Role of TCTG(G/C) direct repeats in cardiac-specific expression

In the present study, we have shown that mutation in the −249bp promoter disrupting the TCTG(G/C) direct repeats led to ectopic expression during development and in the adult, while a mutation disrupting both direct repeats and overlapping GATA site abolished promoter activity. This suggests a novel role for the TCTG(G/C) direct repeats as a critical cis-element in maintaining minimal cardiac-specific expression of the *cTnT* gene. Comparisons of the rat, mouse and human −249bp *cTnT* promoters reveals 87.3–88.6% sequence identity between rodent and human promoters. Particularly, a 97.5% sequence identity is found in the F module of the −249bp *cTnT* promoters (Supplemental Fig. 2A). In addition, the organization of cis-regulatory motifs such as TATA, MCAT, CArG, A/T-rich, MEF2-like, TCTG(G/C) direct repeat and GATA is highly conserved among these organisms (Supplemental Fig. 2B). The chicken promoter also has very similar organization except that it lacks the direct repeats yet gains an Nkx2.5 and extra MCAT sites. It has been shown that −374bp and −268bp chicken promoters are sufficient to drive cardiac restricted expression of reporter genes (Tidyman et al., 2003; Ma et al., 2004). By contrast, the zebrafish promoter possesses a different organization with GATA and Nkx2.5 sites located near the TATA box. Although there is no upstream MCAT, CArG, MEF2-like site or A/T-rich site, a perfect TCTGG direct repeat exists (Supplemental Fig. 2). Interestingly, zebrafish “silent heart” mutant (*sih*^{b109}) is due to a deletion of 13 bp in the region between this TCTGG direct repeats (underline sequences in Supplemental Fig. 2A) of the *cTnT* gene. This change in the promoter led to complete lack of cardiac troponin T protein in the *sih* mutant heart (Sehnert et al., 2002), supporting our hypothesis that the direct repeats and flanking sequences play important roles in *cTnT* expression.

The TCTG(G/C) direct repeats can be also found in other cardiac muscle promoters such as rat *myosin light chain (MLC) 1v* and 2, chicken *MLC2*, mouse *myoglobin*, mouse *α -myosin heavy chain*, mouse *Nkx2.5*, and rat *α -tropomyosin* etc. (Blanchetot et al., 1986; Wieczorek et al., 1988; Shen et al., 1991; Lien et al., 1999). However, the importance of the direct repeats in maintaining cardiac specificity has not been demonstrated in these genes. Previous studies suggest a possible role of the TCTG(G/C) direct repeats as critical elements in driving cardiac gene expression. For example, the mouse *Nkx2.5* upstream enhancer has exactly the same repeats at −9,342bp to −9351bp (TCTGCTCTGG) as that found in the F module of the rat *cTnT* promoter. Interestingly, a GATA site (−9,361bp to −9,365bp) located upstream of this direct repeat has been shown to be important for cardiac expression (Lien et al., 1999), although this GATA does not overlap with the direct repeat like in the F

module. In addition, a direct repeat containing region was found at -9,130bp to -9,154bp (Lien et al., 1999), very much like that found in the D module of the rat *cTnT* promoter. Lein et al. have shown that a region from -9,435bp to -8,922bp (called fragment 11) is necessary for cardiac expression of the *Nkx2.5* at 11.5dpc (Lien et al., 1999). Interestingly, this fragment 11 encompasses both sets of TCTG(G/C) direct repeats. Unfortunately, further dissection of these two sets of direct repeats of the *Nkx2.5* promoter has not been performed. Additionally, the level of transgene expression in the 11.5dpc embryonic heart from this fragment 11 was lower than fragments 8 (-9,700bp to -7,353bp) and fragment 9 (-9,435bp to -7,353bp), suggesting that another cardiac enhancer is located within -8,922bp and -7,353bp. The cardiac enhancer fragment 8, similar to another larger enhancer fragment 5 (-9,700bp to -3050bp), fully recapitulated the temporospatial expression pattern of the endogenous *Nkx2.5* gene through 9.5dpc. However, by 10.5dpc, the transgene expression became non-uniform and restricted to the developing right ventricle (Lien et al., 1999). This is in contrast to the homogeneous expression pattern of the endogenous *Nkx2.5* gene throughout the prenatal and postnatal hearts (Komuro and Izumo, 1993; Lints et al., 1993). The timing of the transgene expression becoming non-uniform is identical to that which we observed for the transgene in the 249LacZ transgenic mice.

The differential expression of HMGB1 in the heart may account for the non-uniform expression of transgene in 249LacZ and F2DLacZ mice

The differential expression of the trans-acting factors that bind the -249bp promoter could, in theory, influence the transgene expression patterns. The consensus sequences for known transcription factors found in the F module of the -249bp promoter includes A/T-rich site, GATA site, MEF2-like site and the novel TCTG(G/C) direct repeats. As reported previously, expression of MEF2C either alone or in combination with GATA4 or *Nkx2.5* did not trans-activate the -497bp promoter activity (Lin et al., 2001). Similarly, expression of MEF2C alone did not trans-activate the -249bp promoter activity (Wang and Lin, unpublished data). Furthermore, we previously showed that MEF2A did not bind to the MEF2-like site in an antibody supershift assay (Wang et al., 2000; Lin et al., 2007). However, the MEF2-like motif was shown to act as another A/T-rich site for the HMGB1/2 binding (Wang et al., 2002). Thus, the differential expression of a MEF2 factor, if any, may have a minimal effect on the nonuniform expression of transgene in transgenic 249LacZ and F2DLacZ mice. Although GATA4 alone moderately enhanced the -497bp promoter activity, strong trans-activation was detected only when both GATA4 and *Nkx2.5* factors were co-expressed (Lin et al., 2007). Since the -249bp promoter does not contain *Nkx2.5*-binding site, the effect of GATA4 factor on the nonuniform expression of transgene may also be a very minimum. Therefore, we investigated the expression of the HMGB1/2 family of proteins. The HMGB1/2 proteins belong to a family of high mobility group non-histone chromatin binding proteins. HMGB1/2 proteins bind A/T-rich regions and cause a bending of the DNA (Grosschedl et al., 1994). This is believed to facilitate the binding of additional proteins such as tissue-specific transcription factors to the DNA through direct binding to DNA and/or by binding the HMGB1/2 protein (Grosschedl et al., 1994; Saito et al., 1999). HMGB1/2 proteins have been shown to interact directly with a number of proteins, including but not limited to, some HOX and POU domain transcription factors and the TATA-binding protein (Zwilling et al., 1995; Zappavigna et al., 1996; Zayed et al., 2003).

The data presented here identified HMGB1 as the major form of HMGB1/2 proteins expressed in heart. Thus, the hypothesis is that the HMGB1 binds to A/T-rich/MEF2-like motif of -249bp *cTnT* promoter, which may facilitate the recruitment of novel 42kDa proteins to the TCTG(G/C) direct repeat site in order for the cardiac-specific expression. In this study, we identify that HMGB1 binds the *cTnT* promoter in vivo. Its expression profile coincides with the 249LacZ and F2DLacZ transgene expression (non-uniform) pattern.

Similar to the HMGB2 (Wang et al., 2002), the HMGB1 also differentially regulates –249bp *cTnT* promoter activity in cultured cardiomyocytes versus non-cardiac cells (data not shown). These results suggest that the HMGB1 regulates *cTnT* expression in vivo and further support that HMGB1 along with the direct repeat-binding proteins (42kDa) provide a way to reinforce the cardiac-specific expression of the *cTnT* gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Robbin Eppinga for his technical assistance. We also thank Norma Sinclair, Trish Yarolem and JoAnn Schwarting from the transgenic core facility at the University of Iowa for the generation of all founder lines used in this study.

Grant sponsor: National Heart, Lung, and Blood Institute, NIH. Grant Number: HL072910

Literature Cited

- Aidinis V, Bonaldi T, Beltrame M, Santagata S, Bianchi ME, Spanopoulou E. The RAG1 homeodomain recruits HMG1 and HMG2 to facilitate recombination signal sequence binding and to enhance the intrinsic DNA-bending activity of RAG1-RAG2. *Mol Cell Biol* 1999;19:6532–6542. [PubMed: 10490593]
- Blanchetot A, Price M, Jeffreys AJ. The mouse myoglobin gene. Characterisation and sequence comparison with other mammalian myoglobin genes. *Eur J Biochem* 1986;159:469–474. [PubMed: 3758071]
- Bodmer R. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development* 1993;118:719–729. [PubMed: 7915669]
- Boonyaratanakornkit V, Melvin V, Prendergast P, Altmann M, Ronfani L, Bianchi ME, Taraseviciene L, Nordeen SK, Allegretto EA, Edwards DP. High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol Cell Biol* 1998;18:4471–4487. [PubMed: 9671457]
- Durocher D, Charron F, Warren R, Schwartz RJ, Nemer M. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *Embo J* 1997;16:5687–5696. [PubMed: 9312027]
- Firulli AB, Olson EN. Modular regulation of muscle gene transcription: a mechanism for muscle cell diversity. *Trends Genet* 1997;13:364–369. [PubMed: 9287492]
- Fishman MC, Olson EN. Parsing the heart: genetic modules for organ assembly. *Cell* 1997;91:153–156. [PubMed: 9346232]
- Franco D, Kelly R, Lamers WH, Buckingham M, Moorman AF. Regionalized transcriptional domains of myosin light chain 3f transgenes in the embryonic mouse heart: morphogenetic implications. *Dev Biol* 1997;188:17–33. [PubMed: 9245508]
- Franco D, Lamers WH, Moorman AF. Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model. *Cardiovasc Res* 1998;38:25–53. [PubMed: 9683906]
- Grosschedl R, Giese K, Pagel J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet* 1994;10:94–100. [PubMed: 8178371]
- Habets PE, Moorman AF, Christoffels VM. Regulatory modules in the developing heart. *Cardiovasc Res* 2003;58:246–263. [PubMed: 12757861]
- Ilagan R, Abu-Issa R, Brown D, Yang Y-P, Jiao K, Schwartz RJ, Klingensmith J, Meyers EN. *Fgf8* is required for anterior heart field development. *Development* 2006;133:2435–2445. [PubMed: 16720880]
- Jiao K, Kulesa H, Tompkins K, Zhou Y, Batts L, Baldwin HS, Hogan BLM. An essential role of *Bmp4* in the atrioventricular septation of the mouse heart. *Genes Dev* 2003;17:2362–2367. [PubMed: 12975322]

- Komuro I, Izumo S. Csx: A murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci USA* 1993;90:8145–8149. [PubMed: 7690144]
- Lee Y, Shioi T, Kasahara H, Jobe SM, Wiese RJ, Markham BE, Izumo S. The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol* 1998;18:3120–3129. [PubMed: 9584153]
- Lien CL, Wu C, Mercer B, Webb R, Richardson JA, Olson EN. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development* 1999;126:75–84. [PubMed: 9834187]
- Lin, JJ-C.; Grosskurth, SE.; Harlan, SM.; Gustafson-Wagner, EA.; Wang, Q. Characterization of cis-regulatory elements and transcription factor binding. In: Zhang, J.; Rokosh, G., editors. *Methods in Molecular Biology: Cardiac gene expression*. Totowa, NJ: Humana Press; 2007. p. 183-201.
- Lin, JJ-C.; Wang, D-Z.; Reiter, RS.; Wang, Q.; Lin, JL-C.; Williams, HS. Differentially expressed genes and cardiac morphogenesis. In: Tomanek, RJ.; Runyan, RB., editors. *Formation of the heart and its regulation*. Boston: Birkhauser; 2001. p. 75-96.
- Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 1993;119:969. [PubMed: 7910553]
- Ma H, Sumbilla CM, Farrance IKG, Klein MG, Insei G. Cell-specific expression of SERCA, the exogenous Ca^{2+} transport ATPase, in cardiac myocytes. *Am J Physiol Cell Physiol* 2004;286:C556–C564. [PubMed: 14592812]
- Reecy JM, Li X, Yamada M, DeMayo FJ, Newman CS, Harvey RP, Schwartz RJ. Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. *Development* 1999;126:839–849. [PubMed: 9895330]
- Ronfani L, Ferraguti M, Croci L, Ovitt CE, Scholer HR, Consalez GG, Bianchi ME. Reduced fertility and spermatogenesis defects in mice lacking chromosomal protein Hmgb2. *Development* 2001;128:1265–1273. [PubMed: 11262228]
- Saito K, Kikuchi K, Yoshida M. The mechanism of sequence non-specific DNA binding of HMG1/2-box B in HMG1 with DNA. *Prot Eng* 1999;12:235–242.
- Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman M, Stainier DY. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat Genet* 2002;31:106–110. [PubMed: 11967535]
- Sepulveda JL, Belaguli N, Nigam V, Chen C-Y, Nemer M, Schwartz RJ. GATA-4 and Nkx2.5 coactivate Nkx2-DNA binding targets: role for regulating early cardiac gene expression. *Mol Cell Biol* 1998;18:3405–3415. [PubMed: 9584181]
- Shen RA, Goswami SK, Mascareno E, Kumar A, Siddiqui MA. Tissue-specific transcription of the cardiac myosin light-chain 2 gene is regulated by an upstream repressor element. *Mol Cell Biol* 1991;11:1676–1685. [PubMed: 1996116]
- Sigmund CD. Major approaches for generating and analyzing transgenic mice. An overview. *Hypertension* 1993;22:599–607. [PubMed: 8406666]
- Sinn HW, Balsamo J, Lilien J, Lin JJ-C. Localization of the novel Xin protein to the adherens junction complex in cardiac and skeletal muscle during development. *Dev Dyn* 2002;225:1–13. [PubMed: 12203715]
- Song L, Yan W, Chen X, deng C-X, Wang Q, Jiao K. Myocardial Smad4 is essential for cardiogenesis in mouse embryos. *Circ Res* 2007;101:277–285. [PubMed: 17585069]
- Tanaka M, Wechsler SB, Lee IW, Yamasaki N, Lawitts JA, Izumo S. Complex modular cis-acting elements regulate expression of the cardiac specifying homeobox gene Csx/Nkx2.5. *Development* 1999;126:1439–1450. [PubMed: 10068637]
- Tidyman WE, Sehnert AJ, Hug A, Agard J, Deegan F, Stainier DYS, Ordahl CP. In vivo regulation of the chicken cardiac troponin T gene promoter in zebrafish embryos. *Dev Dyn* 2003;227:484–496. [PubMed: 12889057]
- Turck F, Zhou A, Somssich IE. Stimulus-dependent, promoter-specific binding of transcription factor WRKY1 to its native promoter and the defense-related gene PcPR1-1 in *Parsley*. *Plant Cell* 2004;16:2573–2585. [PubMed: 15367720]

- Wang DZ, Hu X, Lin JL, Kitten GT, Solursh M, Lin JJ. Differential displaying of mRNAs from the atrioventricular region of developing chicken hearts at stages 15 and 21. *Front Biosci* 1996;1:a1–15. [PubMed: 9159189]
- Wang G, Yeh HI, Lin JJ. Characterization of cis-regulating elements and trans-activating factors of the rat cardiac troponin T gene. *J Biol Chem* 1994;269:30595–30603. [PubMed: 7982978]
- Wang Q, Li-Chun Lin J, Jung-Ching Lin J. A novel TCTG(G/C) direct repeat and an A/T-rich HMG2-binding site control the expression of the rat cardiac troponin T gene. *J Mol Cell Cardiol* 2002;34:1667–1679. [PubMed: 12505064]
- Wang Q, Reiter RS, Huang QQ, Jin JP, Lin JJ. Comparative studies on the expression patterns of three *troponin T* genes during mouse development. *Anat Rec* 2001;263:72–84. [PubMed: 11331973]
- Wang Q, Sigmund CD, Lin JJ. Identification of cis elements in the cardiac troponin T gene conferring specific expression in cardiac muscle of transgenic mice. *Circ Res* 2000;86:478–484. [PubMed: 10700454]
- Warren KS, Lin JL, Wamboldt DD, Lin JJ. Overexpression of human fibroblast caldesmon fragment containing actin-, Ca^{++} /calmodulin-, and tropomyosin-binding domains stabilizes endogenous tropomyosin and microfilaments. *J Cell Biol* 1994a;125:359–368. [PubMed: 8163552]
- Warren KS, Lin JL-C, Wamboldt DD, Lin JJ-C. Overexpression of human fibroblast caldesmon fragment containing actin-, Ca^{++} /calmodulin-, and tropomyosin-binding domains stabilized endogenous tropomyosin and microfilaments. *The Journal of Cell Biology* 1994b;125:359–368. [PubMed: 8163552]
- Wieczorek DF, Smith CW, Nadal-Ginard B. The rat alpha-tropomyosin gene generates a minimum of six different mRNAs coding for striated, smooth, and nonmuscle isoforms by alternative splicing. *Mol Cell Biol* 1988;8:679–694. [PubMed: 3352602]
- Yang J, Drazba JA, Ferguson DG, Bond M. A-kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart. *J Cell Biol* 1998;142:511–522. [PubMed: 9679148]
- Zappavigna V, Falciola L, Helmer-Citterich M, Mavilio F, Bianchi ME. HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J* 1996;15:4981–4991. [PubMed: 8890171]
- Zayed H, Izsvak Z, Khare D, Heinemann U, Ivics Z. The DNA-bending protein HMGB1 is a cellular cofactor of Sleeping Beauty transposition. *Nucleic Acids Res* 2003;31:2313–2322. [PubMed: 12711676]
- Zwilling S, Konig H, Wirth T. High mobility group protein 2 functionally interacts with the POU domains of octamer transcription factors. *EMBO J* 1995;14:1198–1208. [PubMed: 7720710]

Fig. 1.

Diagram of rat *cTnT* promoter. The -497bp proximal promoter of the rat *cTnT* gene contains a basal promoter element composed of TATA box (-34 ~ -24bp), AP2 site (-71 ~ -61bp), M-CAT (-84 ~ -74bp), CARG1 (-140 ~ -129bp) and CARG2 (-191 ~ -182bp), an upstream Nkx2.5 binding site (-382 ~ -375bp) and two similar modules, termed module D (-335 ~ -289bp) and module F (-249 ~ -209bp), each of which contains at least one TCT(G/C) direct repeat (red arrows) and A/T-rich site (red circles). In addition, the F module contains a MEF-2 like motif (yellow box), which also can act as an A/T-rich motif. The -249bp promoter contains F module and a basal promoter element. The 41bp sequence of the F module is shown to diagram the locations of the direct repeats, GATA, A/T-rich site, and MEF-2 like motif, as well as the locations of base pair changes in two mutant promoters, F2D and FCa, derived from the -249bp promoter used in this study.

Fig. 2.

LacZ staining of adult hearts of 497LacZ and 249LacZ transgenic mice. X-gal staining reveals the expression pattern of transgene in the *cTnT* transgenic mice. The 497LacZ mice express the transgene in a uniform pattern throughout the atria and ventricles of the heart (A and B). On the contrary, transgene expression in three different 249LacZ founders is non-uniform with relatively strong expression in the atria and weaker in the ventricles (C-H). The intensity of expression appears strongest in 249LacZ-1 (C and D), weaker in 249LacZ-2 (E and F), and weakest in 249LacZ-3 (G and H). (A, C, E, and G) and (B, D, F, and H) show the ventral and dorsal views of the hearts, respectively. Images are of whole hearts fixed, permeabilized, and stained with X-gal. Scale bar=1mm.

Fig. 3.

LacZ staining of the cryosections of hearts from 497LacZ and 249LacZ transgenic mice. Hearts from transgenic mice were frozen, sectioned, and stained with X-gal to identify transgene staining pattern throughout the heart. The 497LacZ sections show LacZ staining uniformly throughout the entire heart (A). The 249LacZ expression is non-uniform and consistently strongest in the right atrium and interventricular septum (IVS) (B, C, and D). All 249LacZ mice showed similar pattern, with the high expression 249LacZ-1 (B), medium expression 249LacZ-2 (C), and low expression 249LacZ-3 (D) founders shown. A non-transgenic heart does not show any X-gal staining (E). All hearts are representative slides taken from approximately the same region of the heart. Scale bar=1mm.

Fig. 4. Developmental expression pattern of transgene in 497LacZ and 249LacZ transgenic mice. Transgene expression in 497LacZ mice is seen in the heart forming fields at 7.5dpc (A), uniformly throughout the linear heart tube stage at 8.5dpc (B), and uniformly throughout the four chambered heart at 10.5dpc (C). At later stages, transgene expression is detected in the heart, and transiently expressed in the somites and developing skeletal muscle (12.5dpc shown) (D). The 249LacZ founders express the transgene in a similar pattern as 497LacZ mice throughout development, but at reduced levels (E–H). However, at 10.5dpc, expression becomes nonuniform in the developing heart of the 249LacZ mice (G). This non-uniform staining persists throughout development leading to the variegated staining seen in adult transgenic mice. Scale bar in A equals to 50 μ m for A, B, E and F; 200 μ m for C and G; and 0.5mm for D and H.

Fig. 5.

Transgene expression in developing skeletal muscle of 497LacZ and 249LacZ mice. Cross sections of X-gal stained embryos at 12.5dpc were counter-stained with hematoxylin-eosin and then used to closely examine transgene expression profile in the developing skeletal muscle. In the 497LacZ founder, expression is detected in the developing heart (arrowhead), somites, and skeletal muscle (arrow) (A and B). The high expressing 249LacZ-1 founder expresses the transgene in a similar pattern to the 497LacZ founder at this stage, with expression in the heart (arrowhead), somites, and skeletal muscle (arrow) (C and D). However, in the 249LacZ-2 and 249LacZ-3 founders, transgene expression was detected in the developing heart only (E and F). B, D, and F represent high magnification images of A, C, and E, respectively. Scale bar=0.5mm.

Fig. 6. LacZ staining of adult hearts of F2DLacZ transgenic mice. (A and D) and (B and E) represent ventral and dorsal, respectively, views of the hearts. The F2DLacZ transgenic mice have two expression profiles in the adult heart. The F2DLacZ-A founders express the transgene predominantly in the atria, with only faint expression in the ventricles (A–C). Close examination of frozen sections reveals that the only expression in the ventricles is in the IVS (C). The F2DLacZ-AV founders express the transgene in the atria and ventricles of the adult heart (D–F). However, expression is notably stronger in the atria. Close examination of frozen sections shows strong staining in the atria, IVS and RV with no staining in the left ventricle (F). Scale bar=1mm.

Fig. 7.

Ectopic expression of transgene in the brain of adult F2DLacZ-AV mice. The F2DLacZ-AV transgenic mice express the transgene ectopically in the adult brain (C and D), when compared to the 249LacZ transgenic mice (A and B). (A and C) and (B and D) are dorsal and ventral, respectively, views of brain images. All F2DLacZ-AV founders showed ectopic expression of transgene in the brain. Transgene expression in the F2DLacZ-AV brains was found in the olfactory lobes, cerebrum, midbrain and cerebellum. Expression was not detected in the medulla oblongata (D). The staining pattern of the brains was non-uniform, with notable expression of the vasculature system and cerebrum. Scale bar=1mm.

Fig. 8. Developmental expression pattern of transgene in F2DLacZ transgenic mice. The embryos from the F2DLacZ-A founders all express the transgene in the developing heart and somites (A and B), similar to 249LacZ embryos. The embryos from the F2DLacZ-AV founders ectopically express the transgene in the developing embryo when compared to the 497LacZ embryos (C and D). Transgene expression is still detected in the developing heart and somites, yet notable ectopic expression is detected. Stage shown is 11.5dpc when ectopic expression begins to become evident, with ectopic expression continuing throughout the development. Scale bar=0.5mm.

Fig. 9.

HMGB1 is the predominant form of HMGB family of proteins expressed in adult mouse hearts. (A) To test the specificity of anti-HMGB1 and anti-HMGB2 antibodies, Western blot analyses were performed using total protein extracts from atria (lane A) and ventricles (lane V) of wild type mice as well as total protein extracts from uninduced (lane U) and induced (lane I) bacterial pQA1 cells, which are lysogenized with λ gt11 encoding rat HMGB2 fused to β -galactosidase β -gal-HMGB2) (Wang et al., 2002). Rabbit polyclonal anti-HMGB1 (#ab23745, formerly called anti-HMGB1/2) and anti-HMGB2 (#ab11973) antibodies used in this study were generated against synthetic peptides of HMGB1 and HMGB2, respectively (Abcam, Inc., Cambridge, MA). The anti-HMGB2 antibody recognized the

bacterially induced HMGB2 fusion protein but did not detect any protein band from atria and ventricle extracts. On the other hand, the anti-HMGB1 could not recognize HMGB2 fusion protein but detected a protein band with the HMGB1 size from heart extracts. This band is further confirmed as HMGB1 using recombinant protein produced in bacteria (data not shown). (B) Western blot analyses were performed on total protein extracts prepared from skeletal muscle (S.M.), thymus, brain, lung, and heart with anti-HMGB1 and anti-HMGB2 antibodies.

Fig. 10.

HMGB1 expression levels in the atria and ventricles of adult hearts. The expression levels of HMGB1 were determined by quantitative Western blot analysis of protein extracts from atria and ventricles of three different wild type adult mice. The GAPDH expression levels in the same samples were used as a loading control and normalization for quantification. As can be seen in a representative set of Western blot results (the top panel), expression levels of HMGB1 are significantly higher in the atria when compared to the ventricles. The quantitative results shown in the bottom panel reveal that the atria express an average 11-fold higher of HMGB1 proteins than the ventricles ($p=0.001$).

Fig. 11.

HMGB1 expression in the adult heart correlates with transgene expression of the 249LacZ mice. Serial frozen sections were prepared from the 249LacZ mouse heart, with alternating sections used for X-gal staining (A) or immunohistochemistry with anti-HMGB1 antibody (B). The right atrium (RA), right ventricle (RV) and interventricular septum (IVS) have substantial regions that show strong staining with X-gal, indicative of high levels of the transgene expression. These regions also have high levels of HMGB1 proteins, as can be seen in the adjacent section immunostained with an anti-HMGB1 antibody. Conversely, many areas that do not express the transgene, such as the left atria (LA), detection of HMGB1 is very minimal. Scale bar = 1mm.

Fig. 12.

HMGB1 can bind the *cTnT* promoter in vivo. (A) Schematic diagrams of endogenous *cTnT* promoter, -249bp promoter in 249LacZ mice, -497bp promoter in 497LacZ mice and endogenous *mXinα* promoter show their respective transcriptional initiation site (red arrow) and the locations of A/T-rich, MEF2-like and MEF2 sites relative to the primer pairs (a/b, c/d, and e/f) used to amplify chromatin immunoprecipitation (ChIP) products. (B) ChIP was performed on heart nuclear extracts prepared from 497LacZ and 249LacZ transgenic mice using anti-HMGB1 antibody. The anti-HMGB1 antibody pulled down DNA that was able to be amplified by primers (a/b) specific for the endogenous *cTnT* promoter (lane 1) as well as those specific for the transgene promoters (c/d) (lanes 2 and 3 for independent 497LacZ heart nuclear extracts, as well as lane 6 for 249LacZ heart nuclear extract), suggesting that HMGB1 binds to the A/T-rich/MEF2-like sites of the *cTnT* promoter in vivo. However, the anti-HMGB1 antibody could not pull down DNA that was able to be amplified by primer pair (e/f) designed around the MEF2 binding sites of the *mXinα* promoter (lanes 5 and 7), suggesting that the HMGB1 did not bind to MEF2 sites of this promoter. When performed ChIP assay in the absence of the anti-HMGB1 antibody, there was no DNA band that was able to be amplified by either a/b (lane 4) or c/d primer pair (data not shown). However, DNA bands were readily detected by directly amplifying the input chromatin extracts used

in ChIP assays, suggesting the presence of respective chromatin in each extract and the good condition for the PCR amplification.