

Combinatorial *cis*-Acting Elements Control Tissue-specific Activation of the Cardiac Troponin I Gene *in Vitro* and *in Vivo**

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The cardiac troponin I gene is one of the few sarcomeric protein genes exclusively expressed in cardiac muscle. We show here that this specificity is controlled by a proximal promoter (–230/+16) in transfected cardiac cells in culture, in the adult hearts, and in transgenic animals. Functional analysis indicates that MEF2/Oct-1, Sp1, and GATA regulatory elements are required for optimal gene activation because selective mutations produce weak or inactive promoters. MEF2 and Oct-1 transcription factors bind to the same A/T-rich element. A mutation that blocks this binding markedly reduces gene activation *in vivo* and *in vitro*, and overexpression of MEF2A, MEF2C, and MEF2D in noncardiac cells transactivates the cardiac troponin I promoter. Disruption of these elements inactivates the cardiac troponin I promoter in cultured cardiac cells but has a less important role in transfected adult heart. Moreover, nuclear extracts from an almost pure population of adult cardiac cells contain much lower levels of GATA binding activity compared with fetal cardiac cells. These findings point to a differential role of GATA factors in the maintenance of gene expression in the adult heart as compared with the activation of cardiac genes in fetal cardiomyocytes. Overexpression of GATA family members transactivates the cardiac troponin I promoter, and GATA-5 and GATA-6 are stronger transactivators than GATA-4, a property apparently unique to the cardiac troponin I promoter. Transgenic mice carrying the –230/+126 base pair promoter express β -galactosidase reporter gene in the heart both at early stages of cardiogenesis and in the adult animals. These results indicate that the ability of the cardiac troponin I proximal promoter to target expression of a downstream gene in the heart is also maintained when the transgene is integrated into the genome.

Heart formation is characterized by coordinated morphogenetic events and progressive differentiation of the cardiomyocytes. Little is yet known about the genetic pathways that control cardiac morphogenesis, and the molecular bases of the activation of the cardiac differentiation program are even less

understood. Homologous recombination models have recently shown that GATA, MEF2, Nkx 2.5, and HAND family members are major regulatory factors that specify the cardiogenic program and control the first steps of cardiac morphogenesis (1, 2). However, the relative contribution of these factors to cardiogenesis, specification of the cardiac lineage, and maintenance of the differentiation state in the developing and adult heart remain to be established. Cardiac embryogenesis is altered in mice lacking GATA-4, but cardiac cells do form and seem to differentiate properly (3, 4). Targeted disruption of Nkx 2.5 also produces early mortality due to abnormal cardiac development, but cardiac cells are present and express an almost normal pattern of sarcomeric proteins (5). Promoter analyses, on the other hand, have shown that MEF2 and GATA proteins are involved in the regulation of many cardiac genes (6–10). However, the relative contribution of these two proteins varies considerably in different promoters. For example, GATA binding sites are sufficient to activate the B-natriuretic peptide (BNP)¹ gene in cardiac cells (7), whereas a single HF-1b/MEF2 site is crucial to activate the MLC2v gene both *in vitro* (6) and in transgenic animals (11).

We have used the cardiac troponin I (cTNI) gene as a model to investigate the molecular basis of cardiac gene regulation. The cTNI gene encodes for the cardiac specific inhibitory subunit of the troponin complex, one of the few sarcomeric proteins identified to date that is exclusively expressed in cardiac muscle (12–14). In an attempt to investigate the molecular bases of tissue specificity, the mouse homologue was cloned and characterized (15). By progressive deletion of a 4-kilobase pair region, we identified both proximal and distal positive regulatory domains separated by a negative region. However, specific elements responsible for the tissue-specific regulation of the cTNI gene were not characterized. Recently, a GATA element near the transcriptional start site has been shown to regulate the rat cTNI promoter *in vitro* (16); however, it has not been established whether this element is sufficient *per se* for full gene activation and whether other elements are essential for promoter function.

In this study, we focused on the proximal 5'-flanking region of the cTNI gene using three different approaches: *in vivo* transfection through DNA injection into the adult heart, *in vitro* transfection into cultured fetal cardiomyocytes, and generation of transgenic animals. Promoter analyses on cardiac genes have been mostly performed using *in vitro* transfection, but there are a few examples of *in vivo* studies (17–20). Differences between regulation *in vivo* and *in vitro* have been de-

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¹ The abbreviations used are: BNP, B-natriuretic peptide; cTNI, cardiac troponin I; MHC, myosin heavy chain; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; MCK, muscle creatine kinase; MLC2v, myosin eight chain.

scribed for the α -MHC gene (18); however, the specific elements responsible for this differential response have not been identified. We report here that a short segment (-230/+16) of the proximal cTNI promoter is sufficient to confer tissue specificity to a reporter gene in both systems. Functional analyses and protein binding activities demonstrate that functional GATA, Sp1, and MEF2/Oct-1 elements are required for optimal gene activation. However, the specific contribution of these GATA elements varies in fetal cardiac cells *in vitro* as compared with terminally differentiated cardiac cells *in vivo*. GATA binding activity to the cTNI promoter is clearly down-regulated in the adult heart, as we demonstrate here by gel shift analysis using nuclear extracts from an almost pure population of adult cardiomyocytes.

Analysis of the cTNI promoter has also been performed in transgenic mice. Strong activation of the cTNI/*lacZ* transgene was detected in the heart both at early developmental stages and in the adult animals. Moreover, expression of the transgene at early developmental stages resembles that of the endogenous gene, thus indicating that the proximal promoter contains information to specify cTNI gene expression in the heart and that this property is maintained when integration into the genome occurs.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Constructs -230/+16, -145/+16, and -127/+16 were obtained by cloning the corresponding PCR fragments into *Hind*III and *Sal*I sites of pCAT-Basic plasmid (Promega, Madison, WI). Constructs A/T-rich m and G3 m were obtained by introducing specific mutations within the corresponding A/T-rich and GATA site G3 of the -230/+16 construct. Simultaneous mutation of the GATA elements G1 and G2 was obtained by double PCR as described by Naidu *et al.* (21). Briefly, in constructs (G1m/G2m/G3m)I and (G1/G2/G3)mII, nucleotides -57, -59, -64, and -66 of the promoter were replaced using either (G1m/G2m)I sense primer 5'-CCCTGTTATGGCTTGTGCTGG-G-3' or (G1/G2/G3)mII sense primer 5'-CCCTGTTGGCGCTTGGCCTGGG-3' and a G3m antisense primer 5'-TAGTCGACCTCACTGAGGACACTGAGTGTAGG-3' containing a *Sal*I site in its 5'-end. A second series of reactions was carried out with a 5'-upstream primer 5'-T-AGTCGACCTCACTGAGGACACTGAGAT-3' containing a *Hind*III site in the 5'-end and the complementary (G1m/G2m)I or (G1m/G2m)II primer. Each PCR product was subsequently gel-purified and combined in an equimolar ratio, and three cycles of PCR were performed without primers. Finally, a further set of PCR cycles was performed with the addition of *Taq* polymerase and both 5'-upstream and 3'-downstream primers. The final amplified product was digested with *Hind*III and *Sal*I, gel-purified and cloned in pCAT basic. To test basal promoter activity, the cTNI wild type -230/+16 construct and the A/T-rich mutant were cloned into the *Hind*III and *Sal*I sites of the pCAT enhancer plasmid (Promega). All constructs were confirmed by sequencing. Oligonucleotides used for promoter constructs are listed in Table I.

Tissue Cultures and in Vitro Transfection—Cardiac cells were harvested from embryonic day 20–21 rats. Hearts were washed in serum-free medium, dissected free of great vessels and atria, and digested enzymatically as described previously (15). When no further discrete pieces of tissue were visible, cells collected from all cycles were spun out of trypsin, resuspended in medium, and filtered through a nylon mesh. Cells were replated for 45 min to remove nonmuscle cells and plated at $1.5 \times 10^6/60$ -mm plate. 3T3NIH cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics, as described previously (15).

Cardiac cells and 3T3NIH cells were transfected with 5 μ g of reporter gene and 500 ng of cytomegalovirus β -galactosidase vector, as internal control. For transactivation experiments, 1.4×10^5 3T3NIH cells were transfected with 12 μ g of reporter plasmid; 0.2, 2, or 4 μ g of expression plasmid; and 1 μ g of cytomegalovirus β -galactosidase vector, as internal control. In all cases, cells were exposed to the transfection mixture for 16 h before changing the medium. Cells were allowed to express transfected DNAs for 48 h and were then harvested and lysed by three freeze/thaw cycles. Chloramphenicol acetyltransferase activity and β -galactosidase activity were assayed by TLC scintillation counts and spectrophotometric assay, respectively (22). All experiments were repeated with at least two preparations of DNA and were performed at least three times to ensure reproducibility. Statistical analysis was

performed with the Excel program to assess significance of the values.

Direct DNA Injection and Reporter Gene Assays—Plasmid constructs were transfected *in vivo* by direct injection of the DNA into the left ventricular wall essentially as described in Ref. 23, with minor modifications. In brief, adult male Wistar rats weighing between 250 and 270 g were anesthetized by intraperitoneal administration of ketamine (120 mg/kg). Left thoracotomy was performed to expose the beating heart, and 50 μ g of plasmid DNA in 50 μ l of saline solution (0.9% NaCl) was injected with a 30-gauge needle into the apex of the left ventricle. The heart was replaced in the chest, and the incision was closed. 6 days later, the rats were sacrificed, and the hearts were removed. The atria and the great vessels were trimmed, and the ventricles were washed in iced saline and weighed. Ventricles were homogenized in iced cold lysis solution containing 25 mM glycylglycine (pH 7.8), 15 mM MgSO₄, 4 mM EGTA (pH 7.8), 1 mM dithiothreitol (added at the time of use) in a volume proportionate to the mass of the tissue (volume in μ l = $1.5 \times$ mass in mg). Tissues were homogenized with an Ultra Turax T25 by 4–6 strokes of 10 s each. The homogenates were centrifuged at 10,000 rpm for 30 min at 4 °C; the supernatants were quick frozen in liquid nitrogen and stored at -80 °C. Chloramphenicol acetyl transferase activity was assayed by TLC scintillation counts (22). Initial experiments of cotransfection with a luciferase-expressing vector as internal control gave low levels of CAT reporter activity, suggesting possible interference between promoters. Therefore, the internal control was omitted, and multiple independent determinations using different preparations of DNA were used to control for reproducibility of the results. To ensure consistency between experiments, the SV40 promoter and the cTNI -230/+16 promoter were included in every experiment as controls. CAT activities were normalized for protein content. Statistical analysis was performed to assess significance of the values.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from cultured cardiac cells and 3T3NIH cells as follows. Cells were washed twice with relaxation buffer I (100 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 5 mM sodium pyrophosphate, pH 6.8, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min at room temperature, and twice with relaxation buffer II (50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM sodium pyrophosphate (pH 6.8), and 0.5 mM phenylmethylsulfonyl fluoride) under the same conditions. All of the solutions used in this protocol were supplemented with antiproteases at 2 mg/ml final concentration. Cells were equilibrated with solution A (10 mM Hepes, 10 mM KCl, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 3 min at room temperature. To release nuclei, cells were coated with the same solution containing 0.25% Nonidet P-40 Nonidet at room temperature for 5 min, scraped, transferred into a Dounce homogenizer, and disaggregated by mechanical homogenization. The release of nuclei was checked under the microscope, and nuclei were pelleted by centrifugation at 2000 rpm for 10 min in a refrigerated centrifuge. The pellet was resuspended in solution A containing 0.25% Nonidet P-40 and centrifuged as before. Nuclei were gently resuspended in extraction buffer (20 mM Hepes, pH 7.9, 0.45 NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and incubated for 1 h at 4 °C in a rotating chamber. The samples were centrifuged at 35,000 rpm for 1 h to pellet the DNA and the microsomal fraction, and the supernatant was stored at -80 °C in aliquots.

Nuclear extracts were also prepared from adult cardiomyocytes purified from noncardiac cells essentially as described in Ref. 24. Briefly, adult rat hearts were retrogradely perfused through aorta with Ca²⁺-free buffer (116.4 mM NaCl, 5.4 mM KCl, 1.6 mM MgSO₄, 26.2 mM NaHCO₃, 1.0 mM NaH₂PO₄, 5.6 mM D-glucose) equilibrated with 95% CO₂ and 5% O₂ (pH 7.36 \pm 0.05) at 37 °C. The perfusate was then switched to a similar solution containing 0.5 mg/ml collagenase B for about 20 min. This incubation softened the heart tissue, thus facilitating its mincing. The resulting cardiomyocyte suspension was filtered through a nylon mesh, and the cells were allowed to sediment. The supernatant was removed by aspiration, and the pellet was resuspended with relaxation buffer I for isolation of nuclei. The contamination of noncardiac cells was less than 7%, a value in agreement with previous data (24).

DNA/protein reactions and high resolution electrophoretic mobility shift assays (EMSA) were performed as described in Ref. 25, using 0.3 ng of radiolabeled probe, 1 μ g of poly(dI-dC) and a 50–100-fold excess of competitor DNA. 2 or 4 μ g of nuclear extracts were used in all reactions, except for adult heart nuclear extracts, which were tested at increasing concentrations. Supershift assays were performed with antibodies specific for MEF2A, MEF2B, MEF2D (26), Oct-1 (27), GATA-4, and Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The antibodies were added to the DNA/nuclear extracts mix, and the reactions were allowed to proceed for 20 min before loading the samples. Oligonucleotides used

for these experiments are listed in Table I.

Generation of cTNI/lacZ Transgenic Mice—The cTNI transgene was obtained by cloning the $-230/+126$ fragment of the promoter in pSKlacZ-SV40 poly(A) vector carrying a nuclear sequence. The resulting transgene was excised at the *Xho*I and *Not*I sites, gel-purified, and passed through a Qiagen column. Transgenic mice were generated by microinjection of purified insert into fertilized FVB \times BDF1 eggs at a concentration of 500 copies/pl using standard techniques (28). Injected eggs were reimplanted into pseudopregnant (C57 \times CBA) foster mothers. To screen for positive lines, DNA was prepared from mouse tails or toes and was analyzed by PCR and Southern blotting according to standard techniques (22).

Hemizygous transgenic males from the F1 generation were crossed with nontransgenic females (BDF1 or CD1). Embryos were dated taking 0.5 days postcoitum as the day of vaginal plug, and they were dissected in $1\times$ phosphate-buffered saline, fixed in 4% paraformaldehyde for periods of 5–45 min, rinsed in $1\times$ phosphate-buffered saline, and stained in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside solution at 37 °C for periods from 2 h to overnight (29). Embryos were maintained in phosphate-buffered saline and photographed with an Olympus SZH10 stereoscope.

Whole Mount *In Situ* Hybridization—To assess expression of the endogenous cTNI gene, embryonic day 9.5 embryos were processed for whole mount *in situ* hybridization essentially as described previously (30). An 800-base pair DNA fragment corresponding to the rat cTNI clone (12) was used to produce sense and antisense RNAs. RNA was synthesized to incorporate digoxigenin-UTP (Boehringer Mannheim) according to the manufacturer's instruction. Dissected embryos were fixed in 4% paraformaldehyde, treated with proteinase K (10 μ g/ml) for 10 min, and incubated with the probes at a concentration of 1 μ g/ml. Incubation with the antidigoxigenin antibody (Boehringer Mannheim) was performed according to the manufacturer's instructions. Labeling was revealed in the presence of BM Purple AP substrate (Boehringer Mannheim) after a 2-h incubation. Embryos were washed in phosphate-buffered saline-Tween 20 several times and photographed as described above.

RESULTS

GA-rich, A/T-rich, and GATA Elements Are Required for cTNI Gene Regulation

Sequence analysis of the mouse cTNI promoter region from -230 to $+16$ revealed several putative protein binding sites, including GA-rich sequences, two of which are indicated as S1 and S2; GATA elements indicated as G1, G2, and G3; and an A/T-rich element (Fig. 1A). Two GATA elements (G1 and G2) lie closely associated between -67 and -57 of the promoter, whereas G3 lies at -7 , just upstream of the gene transcriptional start site. Two GATA elements are also present in the rat (16) and human (31) gene, approximately at the same positions. The A/T-rich element lying at position -36 of the promoter has 90% homology with the MEF2 consensus 5'-CTA(A/T)₄TAA/G-3' (32) and 50% homology with the Oct-1 consensus 5'-ATG-CAAAT-3' (33) and represents a noncanonical TATA box. An identical A/T-rich element is present in the rat (16) and the human (31) cTNI gene.

We used deletion and mutation analyses to assess the contribution of these *cis*-acting elements and their relative transcription factors to gene activation (Fig. 1B). Each construct was tested *in vitro* in fetal cultured cardiac cells and *in vivo* by direct DNA injection into the left ventricular wall of adult hearts. Results of transfections are summarized in Fig. 2. The role of the GA-rich sequences S1 and S2 was analyzed by producing constructs $-145/+16$ and $-127/+16$, which contain only one or no GA-rich element. These deletions progressively reduced *in vitro* promoter activity (Fig. 2A), but only deletion of S2 had some effect *in vivo* (Fig. 2B).

The contribution of GATA elements to tissue specificity and efficiency of expression was determined by mutation of G1, G2, and G3. Sequence GATA was changed into CACA in the first sets of constructs (mI). Mutation in G1/G2 and G3 reduced activity *in vitro* but had a weak effect or no significant effect *in vivo*. As expected, simultaneous mutation of G1, G2, and G3

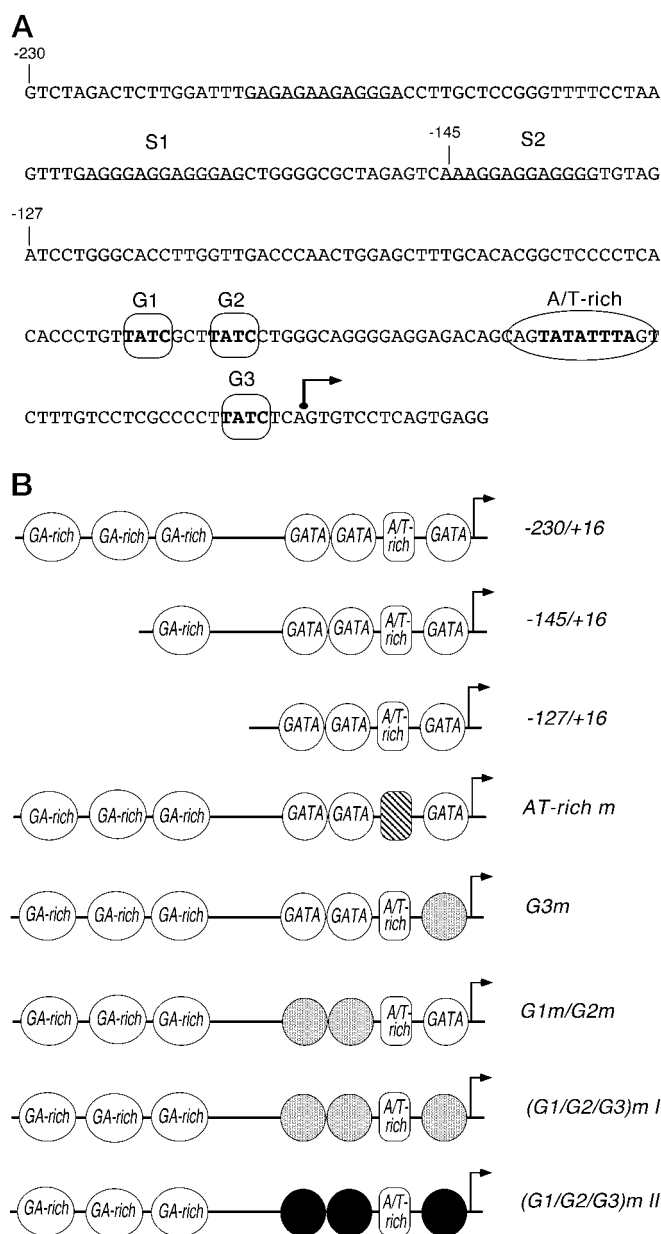


FIG. 1. The cTNI promoter $-230/+16$ contains multiple putative protein binding sites. A, sequence analysis revealed the presence of several protein binding sites in the proximal promoter region $-230/+16$. These include three GA-rich sequences, two of which (S1 and S2) are putative Sp1 binding sites; three GATA elements (G1, G2, and G3); and an A/T-rich sequence. The dashed arrow indicates the transcriptional initiation site of the gene. B, schematic representation of the deleted and mutated constructs analyzed in this study. Mutated elements are represented as striped, shaded, or black circles or boxes. Black and shaded circles indicate two different mutations introduced in the three GATA elements of the promoter. The abbreviations used to identify each construct are indicated on the right.

had a dramatic effect *in vitro* but a weak effect *in vivo* (Fig. 2). Similar results were obtained by introduction of a second type of mutation that changed sequence GATA into GCCA (mII) (Fig. 2). Thus, GATA elements control cTNI gene expression but with substantial quantitative differences *in vitro* in fetal cultured cardiac cells and *in vivo* in adult hearts.

Mutation of the cTNI A/T-rich element also produced a greater reduction of activity *in vitro* than *in vivo* (Fig. 2, A and B). The cTNI A/T-rich element is a noncanonical TATA box potentially able to bind the transcription factor IID. To determine that mutation in this site did not alter basal promoter

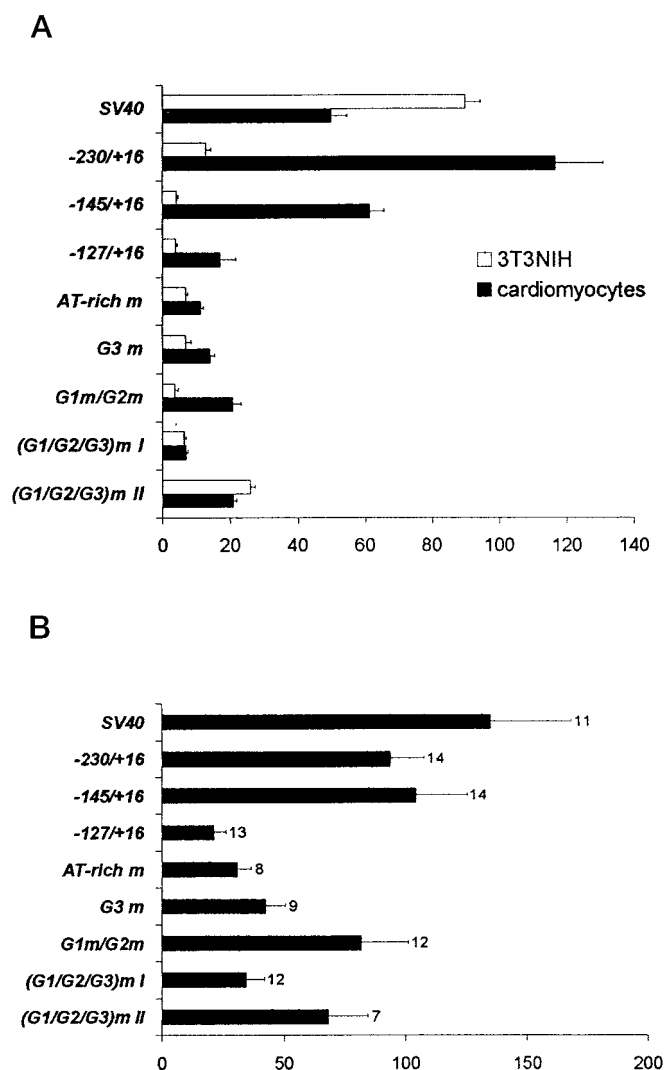


FIG. 2. Functional role of cTNI cis-acting elements *in vitro* and *in vivo*. A, cTNI constructs were transfected in cardiac cells and 3T3NIH cells. Values are expressed as -fold increase over the promoterless construct, whose activity accounted for 3.02 ± 0.47 in 3T3NIH cells and 2.06 ± 0.17 in cardiac cells. Values represent ((CAT activities/ β -galactosidase activity) $\times 100$) \pm S.E. Each construct was tested in a minimum of four independent determinations for 3T3NIH cells and in a minimum of six independent determinations for the cardiac cells. B, the same cTNI constructs were transfected in adult rat hearts. Values are expressed as -fold increase over the promoterless construct, whose activity accounted for 0.16 ± 0.025 . Values represent ((CAT activities/protein content) $\times 100$) \pm S.E. CAT activity and protein content were tested using 5% of homogenate. Numbers of independent determinations are indicated for each construct.

activity, we cloned the wild type promoter $-230/+16$ and the A/T-rich mutant in a pCAT Enhancer vector carrying the SV40 enhancer downstream of the CAT reporter gene and performed a functional test in 3T3NIH cells. The A/T-rich mutation did not affect basal promoter activity, which was even higher compared with the wild type promoter. Normalized CAT values of the promoterless enhancer CAT, the $-230/+16$ enhancer CAT, and A/T-rich m Enhancer CAT were 4.5 ± 0.3 , 66.4 ± 2.7 and 210.8 ± 16.1 , respectively. Thus, an A/T-rich element controls cTNI gene expression over its basal transcription levels both *in vitro* in fetal cultured cardiac cells and *in vivo* in the adult heart.

Mutation of the GA-rich, A/T-rich, and GATA elements of the cTNI promoter was also tested in 3T3NIH cells, where the wild type construct is constitutively expressed at very low levels (Fig. 2A). We observed that each mutation slightly reduced

TABLE I
Oligonucleotides used in this study

Mutated nucleotides are underlined. Asterisks indicate oligonucleotides used for EMSA but not for mutant constructs. Oligonucleotides specific for this purpose are indicated under "Experimental Procedures."

Oligonucleotide ^a	Sequence (sense strand)
G1/G2	5'-CGCCTGTTATCGCTTATCCTGGG-3'
G1m/G2	5'-CGCCTGTTGTGGCTTATCCTGGG-3'
G1/G2m	5'-CGCCTGTTATCGCTTGTGCTGGG-3'
G1m/G2m*	5'-CGCCTGTTATGGCTTGTGCTGGG-3'
G3	5'-CGCCCCTTATCTCAG-3'
G3m*	5'-CGCCCCTTGTCTCAG-3'
BNP90 (7)	5'-GATCCAGGAATGTGCTGATAAATCAGAGAT-AACCCA-3'
Oct-1 (53)	5'-GTACCTATTTCGATAAAGC-3'
S1	5'-CGAGGGAGGAGGGAG-3'
S2	5'-CGTTTGAGGGAGGAGGGAG-3'
Sp-1 (33)	5'-CTTCCGTTGGGGCGGGCTTACAG-3'
A/T-rich	5'-CGCAGTATATTAGTCTG-3'
A/T-rich m	5'-CGCAGTATATTAGCTCTG-3'
MCKMEF2 (32)	5'-TCGCTCTAAAATAACCCCTG-3'
MLC2vMEF2 (6)	5'-GATCTCCCTGGGGTTAAAAATAACCCCA-3'

^a References for the oligonucleotide sequences are indicated in parentheses.

promoter activity and that this reduction was higher with deletion of the two GA-rich elements and with (G1/G2/G3)mI mutant. A modest but statistically significant increase of activity was obtained with (G1/G2/G3)mII mutant.

Nuclear Proteins MEF2, Oct-1, Sp1, and GATA Bind to the Proximal cTNI Promoter

Transient expression analyses indicate that GA-rich elements, GATA elements, and the A/T-rich element are required for optimal expression of the cTNI promoter in cardiac cells. To characterize transcription factors binding to these sequences, we used EMSAs with DNA probes encompassing the potential *cis*-regulatory elements and nuclear extracts from cultured cardiac cells. Oligonucleotide probes and competitors used in this study are shown in Table I.

Factors Binding to the cTNI GA-rich Elements—Protein binding to the cTNI GA-rich sequences was assessed by using probes corresponding to elements S1 and S2. Nuclear extracts from cultured cardiac cells produced a major shifted complex (Fig. 3, lanes 1 and 7). This complex was efficiently self-competed (lanes 2, 3, 8, and 9), was removed by the addition of a canonical Sp1 target sequence (lanes 4, 5, 10, and 11), and was supershifted by an anti-Sp1 antibody (lanes 6 and 12). Additional complexes were observed by using probe S2 and were competed off by a canonical Sp1 sequence. Supershift by the anti-Sp1 antibody (lane 12) removed the major complex without affecting the others. To elucidate further the nature of the other complexes, antibodies specific for Sp2, Sp3, and Sp4 were tested. The anti-Sp3 antibody (Fig. 3, lane 15) removed one of these bands and gave a slight supershift, whereas no change was observed with the anti-Sp2 and -Sp4 antibodies (lanes 14 and 16). No Sp1 binding activity was observed with an oligonucleotide corresponding to the GA-rich sequence at $-212/-200$ of the promoter (not shown).

Factors Binding to the cTNI A/T-rich Element—EMSA with the cTNI A/T-rich element and cardiac nuclear extracts gave a more prominent complex and a weaker slow migrating band (Fig. 4A, lane 1). Both complexes were self-competed (lanes 2 and 3). The slower migrating complex was completely removed by the addition of either the MCK MEF2 site (lanes 4 and 5) or the MLC2v MEF2 site (lanes 6 and 7). The faster migrating complex, on the other hand, was efficiently removed by the addition of a canonical Oct-1 sequence (lanes 10 and 11) but not by the canonical MCK MEF2 nor by the MLC2v MEF2 sites.

FIG. 3. cTNI GA-rich motifs are target for Sp1. Nuclear extracts from cultured cells (4 μ g) were mixed with a double-stranded oligonucleotide corresponding to the cTNI GA-rich elements S1 (lane 1) and S2 (lane 7). Competitions were carried out with 50- and 100-fold molar excess of self-competitor (lanes 2, 3, 8, and 9) and a canonical Sp1 sequence (lanes 4, 5, 10, and 11). An antibody that recognizes Sp1 (Sp-1 Ab) supershifted the Sp1 band both with S1 (lane 6) and S2 (lane 12) probes. Antibodies specific for Sp2 (Sp-2 Ab, lane 14), Sp3 (Sp-3 Ab, lane 15), and Sp4 (Sp-4 Ab, lane 16) were also tested with the S2 probe. The position of the Sp1 binding activity is indicated by arrows. A dash indicates the position of complexes supershifted by Sp1 and Sp3 antibodies.

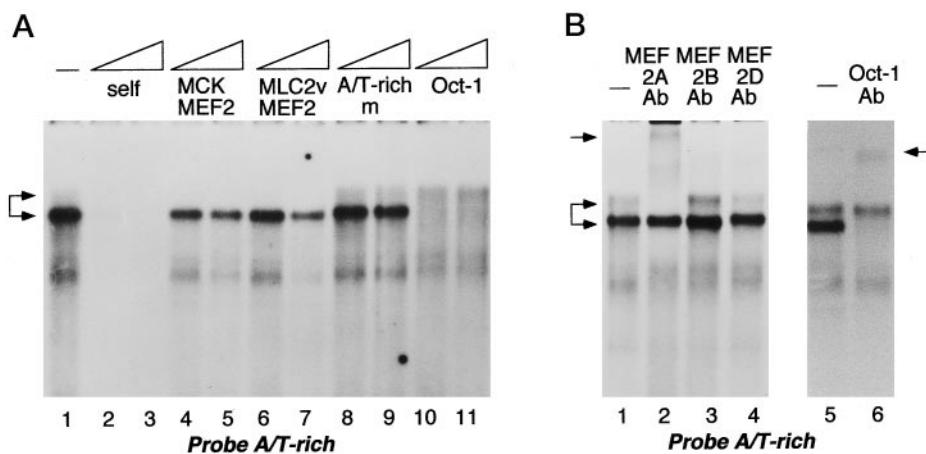
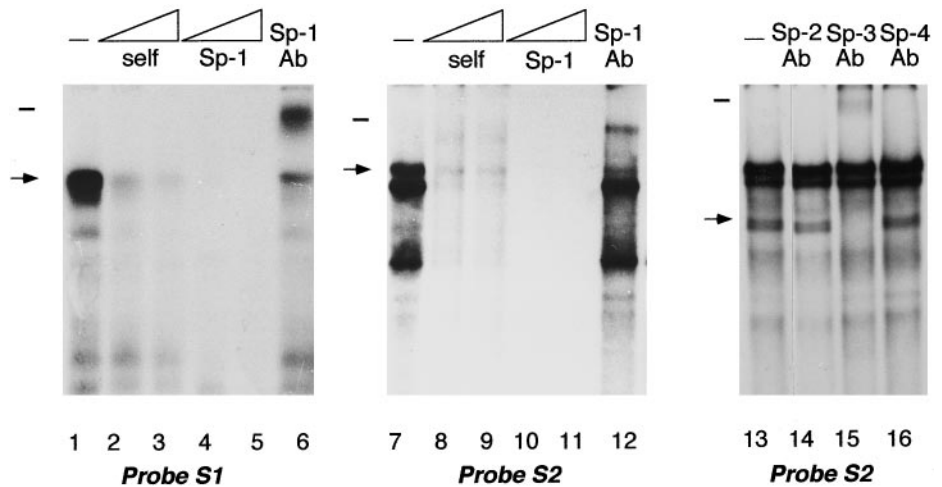


FIG. 4. The cTNI A/T-rich element is target for MEF2 and Oct-1 transcription factors. **A**, cardiac nuclear extracts from cultured cells (4 μ g) were mixed with a double-stranded oligonucleotide corresponding to the cTNI A/T-rich element. Two major complexes, indicated with arrows to the left, were shifted (lane 1). Both complexes were removed by the addition of 50- and 100-fold molar excess of self-competitor (lanes 2 and 3). The slowly migrating complex was removed by the addition of oligonucleotides corresponding to MCK MEF2 (lanes 4 and 5) and MLC2v MEF2 (lanes 6 and 7). This complex was not removed by the addition of an oligonucleotide containing the mutated cTNI A/T-rich element (A/T-rich m) (lanes 8 and 9). The faster migrating complex, on the other hand, was removed by the addition of an oligonucleotide specific for Oct-1 (lanes 10 and 11). **B**, nuclear extracts from cardiac cells (4 μ g) were tested with the cTNI A/T-rich element in the absence of antibody (lanes 1 and 5) and in the presence of antibodies specific for MEF2A (lane 2), MEF2B (lane 3), MEF2D (lane 4) and Oct-1 (lane 6). The slowly migrating complex was supershifted by an anti-MEF2A antibody, while the faster migrating complex was supershifted by an anti-Oct-1 antibody (arrows).

Neither complex was removed by the A/T-rich m mutant (lanes 8 and 9). The slower but not the faster migrating complex was supershifted by an antibody specific for MEF2A but not by antisera against MEF2B and MEF2D (Fig. 4B, lanes 2–4). The faster migrating complex, on the other hand, was supershifted by an anti-Oct-1 antibody, which, in turn, had no effect on the MEF2 complex (Fig. 4B, lane 6). The addition of nonimmune sera as a control did not interfere with the binding (not shown), thus indicating the specificity of the protein-antibody interactions. In conclusion, the A/T-rich sequence of the cTNI promoter is a complex site that binds at least two transcription factors, MEF2 and Oct-1. Binding of MEF2 factor(s) to the cTNI promoter was rather weak, whereas a strong signal was observed by testing the same nuclear extracts with either a consensus Oct-1 or a MEF2 site (not shown).

Factors Binding to the cTNI GATA Elements—Oligonucleotide probes containing GATA element G1/G2 and GATA element G3 gave a strong shift when incubated with nuclear extracts from cultured cardiac cells (Fig. 5A, lanes 1 and 11). This complex was readily removed by the self-competitors (lanes 2, 12, and 13) and by GATA sequences from the BNP (lanes 9, 10, 14, and 15). In contrast, it was not removed by G1m/G2m and G3m mutants (lanes 7, 8, 16, and 17).

GATA elements G1 and G2 are closely associated in the cTNI sequence and potentially able to cooperate for protein binding. To examine binding preferences of the GATA complex for G1 and G2, mobility shift to G1/G2 probe was competed with an oligonucleotide containing either mutated G1 and intact G2 (G1m/G2) or mutated G2 and intact G1 (G1/G2m). G1m/G2 competed weakly for protein binding activity (Fig. 5A, lanes 3 and 4), whereas G1/G2m blocked complex formation even at the lowest concentration (lanes 5 and 6). These results suggest that integrity of both GATA elements is required for maximal binding and, nevertheless, that G1 and G2 differ, at least for protein-DNA affinity.

The nature of the cTNI GATA binding activity was further explored by supershift with an anti GATA-4 antibody (Fig. 5B). Complex obtained with G1/G2 probe and G3 probe was partially supershifted (lanes 2 and 6), whereas the addition of nonimmune serum gave no effect (lanes 3 and 7). Excess antibody (4 μ l) in the reactions did not change the level of supershift, thus suggesting that factors other than GATA-4 probably contribute to the complexes.

Our functional analysis showed that mutations within the cTNI GATA elements have a strong effect *in vitro* in fetal cultured cardiac cells but less effect *in vivo* in the adult heart.

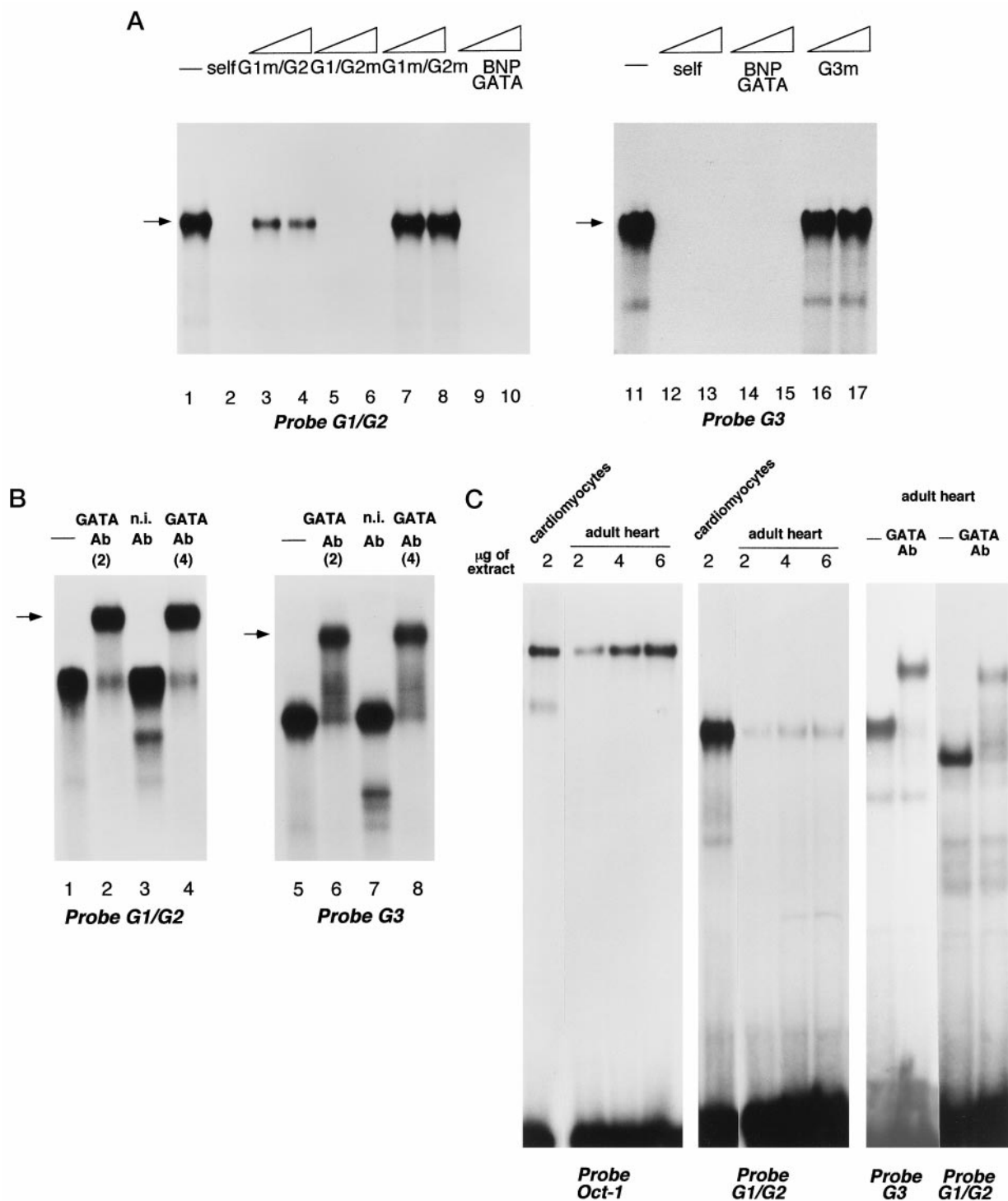


FIG. 5. The cTNI GATA elements bind GATA transcription factor(s). The cTNI GATA binding activities are down-regulated in the adult heart. **A**, nuclear extracts (4 μ g) from cultured cardiomyocytes were probed with a double-stranded oligonucleotide corresponding to either element G1/G2 or element G3 of the cTNI promoter. A strong complex was shifted in both cases (lanes 1 and 11) and was removed by the addition of 50- and 100-fold molar excess of self competitor (lanes 2, 12, and 13) and BNP GATA (lanes 9, 10, 14, and 15). In contrast, the mutated G1m/G2m (lanes 7 and 8) and G3m (lanes 16 and 17) oligonucleotides did not remove this complex. G1/G2m competed efficiently for binding (lanes 5 and 6), whereas G1m/G2 had little effect (lanes 3 and 4). **B**, 2 and 4 μ l of an antibody that recognizes GATA-4 (GATA Ab) gave a strong but partial supershift of the GATA complex both with G1/G2 (lanes 2 and 4) and G3 probes (lanes 6 and 8). The addition of the nonimmune serum (n.i. Ab) had no effect (lanes 3 and 7). Note that two different concentrations of the specific antibody (2 and 4 μ l) gave the same partial supershift. The supershifted band is indicated by arrows. **C**, nuclear extracts from cultured fetal cardiac cells and adult rat cardiomyocytes were tested with Oct-1 and G1/G2 probes. Labeled probes were adjusted to equal specific activity. Increasing amounts (2, 4, and 6 μ g) of adult nuclear extracts were compared with the signal obtained with 2 μ g of nuclear extracts from cultured cardiac cells. Note that even at the highest protein concentration adult nuclear extracts gave a barely detectable signal for GATA, whereas a strong signal for Oct-1 was detected with an Oct-1-specific probe. 24 μ g of adult nuclear extracts were also tested with G3 and G1/G2 probes and the anti-GATA-4 antibody. The antibody supershifted most, but not all, of the GATA complex.

We asked whether different protein-DNA interactions or different levels of the same binding activity were detectable in nuclear extracts from adult hearts as compared with cultured

cardiac cells. Probe G1/G2 was tested by EMSAs using constant amounts of nuclear extracts from cultured cells (2 μ g) and increasing amounts (2, 4, and 6 μ g) of nuclear extracts obtained

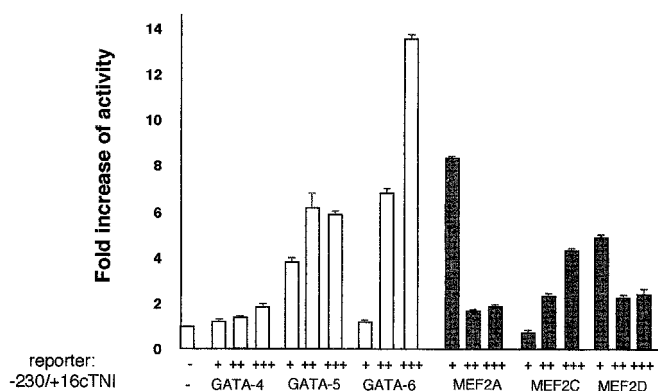


FIG. 6. GATA and MEF2 factors transactivate the cTNI promoter in 3T3NIH cells. 3T3NIH cells were transfected using the $-230/+16$ cTNI reporter gene and various amounts of GATA-4, GATA-5, GATA-6, MEF2A, MEF2C, and MEF2D expression vectors. All transfections were performed using $12 \mu\text{g}$ of reporter, 0.2 (+), 2 (++) or 4 (+++) μg of expression vector and $1 \mu\text{g}$ of cytomegalovirus β -galactosidase as internal control. Values represent -fold induction and are the average of three independent determinations \pm S.E.

from an almost pure population of cardiomyocytes from adult rat hearts. Identical analysis was performed with an Oct-1 probe, for comparison. As shown in Fig. 5C, cardiac extracts shifted the same GATA complex, but signals were much weaker with adult cardiomyocytes. On the other hand, signals obtained with the Oct-1 probe were similar, suggesting that low GATA activity in the adult heart was not due to poor quality of nuclear extracts.

The low GATA binding activity in adult cardiomyocytes was tested with the anti GATA-4 antibody. $24 \mu\text{g}$ of extracts were tested with probe G3 and probe G1/G2 in the presence of excess antibody. The complex was only partially supershifted in both cases, again suggesting that factors other than GATA-4 may contribute to the complex.

GATA and MEF2 Factors Transactivate the cTNI Gene

The functional role of GATA elements was further tested by transactivation of the cTNI promoter in 3T3NIH cells. Cells were cotransfected with the $-230/+16$ promoter and increasing amounts of expression vectors (0.2 , 2 , or $4 \mu\text{g}$ of DNA). Under these conditions, transfection of $2 \mu\text{g}$ of expression plasmid gave a sufficient amount of protein so as to be fully detectable by gel shift analysis (not shown). Overexpression of GATA-4 had a modest transactivation ability. In contrast, GATA-5 and GATA-6 were able to transactivate the promoter to much higher levels up to about 6- and 13-fold increase of activity, respectively (Fig. 6).

Transactivation ability of MEF2 was also tested in 3T3NIH cells, which are known to express Oct-1 but not MEF2 proteins. Overexpression of MEF2A gave the highest transactivation corresponding to an 8-fold increase of activity, whereas a weaker effect was observed with MEF2C and MEF2D (Fig. 6). While transactivation increased with increasing amounts of MEF2C expression vectors, the highest transactivation was observed with the lowest concentration of MEF2A and MEF2D expression vectors.

A Proximal cTNI Promoter Shows Cardiac Specific Expression in Transgenic Mice

In light of the finding that a very short segment of the cTNI promoter was highly active both *in vitro* in fetal cultured cardiac cells and *in vivo* in adult hearts, we tested the minimal promoter in transgenic mice. For this purpose, the same promoter region was fused to the first cTNI exon sequences up to nucleotide $+126$, and the resulting transgene $-230/+126$ was

cloned upstream of a *lacZ* reporter gene containing a nuclear leader sequence. Preliminary transfections in cultured cardiac cells had shown that activity of constructs $-230/+126$ and $-230/+16$ were similar.²

In two independent lines cTNI/*lacZ* transgene was highly expressed both in the embryonic and in the adult heart (Fig. 7, B-C and D-E, respectively). Expression at embryonic day 9.5 was comparable with that of the endogenous gene tested by whole mount *in situ* hybridization using a specific cTNI probe (A). Very short incubation of the transgenic embryos with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside revealed a slightly lower expression in the outflow tract, as previously reported for the endogenous gene both in the rat (34) and in the mouse (35). These results corroborate the data from transient transfection analysis and indicate that the proximal cTNI promoter is able to specify expression of a downstream reporter gene both in the embryonic and adult heart even when integrated into the genome. A detailed analysis of the transgenic lines generated with the proximal cTNI promoter will be presented elsewhere.

DISCUSSION

This study shows that a small cTNI promoter sequence is sufficient to confer cardiac specific expression of reporter genes in transfected cardiac cells *in vitro*, *in vivo*, and in transgenic mice.

At least three different types of protein binding sites, specific for Sp1, MEF2/Oct-1, and GATA, are required for expression of the cTNI promoter in cardiac cells because mutations that abolish one of these elements without affecting the others produce a weak or inactive promoter. However, the relative contribution of each regulatory element to activation differs *in vitro* in fetal cultured cardiac cells and *in vivo* in adult hearts. This finding points to a differential role of transcription factors for activation of the cTNI gene in proliferating fetal cardiomyocytes as compared with maintenance of gene expression in the adult tissue. It will be interesting to extend this comparative study to other genes, since most promoter studies to date have been based either on *in vitro* or *in vivo* analysis but rarely on both.

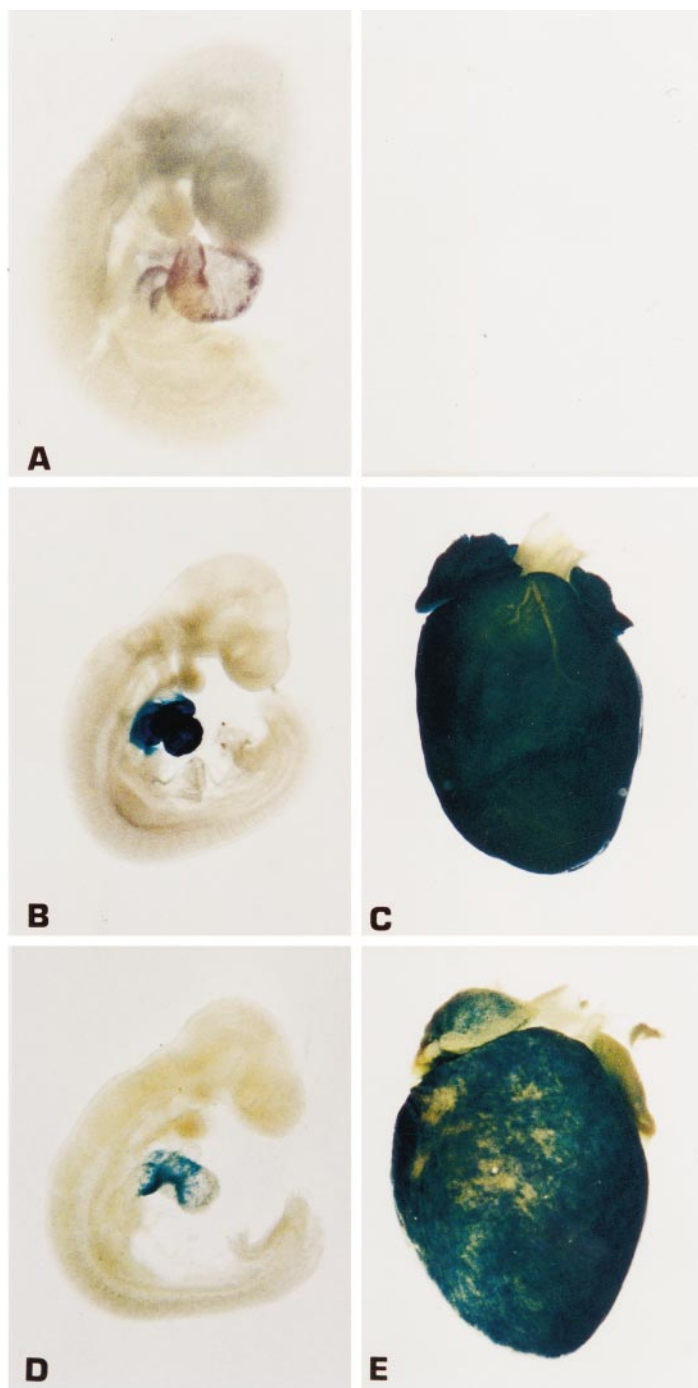
Three GA-rich elements are present in the mouse cTNI promoter between -230 and -127 and are also conserved in the human gene approximately at the same positions (31). GA-rich elements of the human promoter have been interpreted as minisatellite repeats based upon homology with a consensus sequence identified in other human genes (36). However, we show here that the central core sequence of two out of the three elements, indicated as S1 and S2 in Fig. 1, are functional binding sites for the zinc finger transcription factor Sp1 and, in the case of S2, also for Sp3. DNA-bound Sp1 molecules have been implicated in cardiac transcriptional regulation. An Sp1 binding site is necessary to activate the α -cardiac actin promoter in a tissue-specific manner (37) and a complex Sp1 sequence acts as an α_1 -adrenergic response element in the atrial natriuretic factor promoter (38). A more general function of Sp1, however, is the activation of the general transcriptional machinery through coactivators (39, 40). Sp1 recruits the basal transcription factors to core promoters containing either a functional TATA-box or an initiator (reviewed in Ref. 40). Interestingly, the cTNI promoter contains a putative initiator at position $+8$ (TCAGTGT). The question as to whether a TATA- or an initiator-dependent mechanism activates the gene remains to be investigated. Another open question is whether Sp1 is implicated in tissue-specific or basal activation of the cTNI gene. Deletion of the two Sp1 binding sites diminishes

² S. Ausoni, unpublished observation.

FIG. 7. The cTNI minimal promoter drives cardiac specific expression of a lacZ reporter in transgenic mice. cTNI/lacZ transgenic mice were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside as detailed under "Experimental Procedures." Embryonic day 9.5 embryos (*B* and *D*) and adult hearts (*C* and *E*) showed strong and tissue-specific expression. Expression of the endogenous gene was also analyzed in embryonic day 9.5 embryos by whole mount *in situ* hybridization using a cTNI antisense probe (*A*). No reaction was detected with a cTNI sense probe (not shown).

cTNI_{LacZ21}

cTNI_{LacZ43}



transcription of the cTNI promoter in 3T3NIH cells, but differences are not statistically significant.

The cTNI gene contains an A/T-rich element that binds MEF2 and Oct-1 transcription factors, as demonstrated by EMSAs and supershift experiments with specific antibodies. Promoter activity requires integrity of this element because a mutation that selectively abolishes binding of both MEF2 and Oct-1 significantly reduces activation *in vitro* in fetal cultured cardiac cells and, to a lesser extent, *in vivo* in adult hearts. An additional, somewhat intriguing observation, is that the cTNI MEF2/Oct-1 binding site is embedded within a noncanonical TATA-box potentially able to bind the transcription factor IID (41). A similar feature has been found in other muscle-specific genes, such as the myoglobin promoter (42) and the *Xenopus* MyoD promoter (43). Additional studies will be required to establish whether the cTNI A/T-rich element behaves as a

functional TATA and whether MEF2/Oct-1 interacts with elements of the basal transcriptional machinery. Nevertheless, our functional studies demonstrate that when we block MEF2/Oct-1 binding we produce a weak or inactive promoter without affecting basal transcription. Therefore, tissue-specific activation of the cTNI gene is governed by MEF2/Oct-1. The role of MEF2 has been investigated in more detail by transactivation experiments. Overexpression of MEF2A, MEF2C, or MEF2D activates the cTNI promoter in 3T3NIH cells, which constitutively express Oct-1 but not MEF2 proteins. Therefore, it is likely that tissue-specific activation of the cTNI gene is dependent upon MEF2 binding to the A/T-rich element.

Three GATA elements are present in the cTNI proximal promoter and are targets for GATA-4 transcription factor. At present, we cannot exclude the possibility that GATA-5 and GATA-6 or other GATA-like factors also bind to these targets.

In fact, we were never able to supershift completely the GATA complex, even in the presence of an excess of anti-GATA-4 antibody. GATA elements G1 and G2 are closely associated, a feature that recalls other GATA-dependent promoters such as the cardiac/slow cTNC (10), the α -MHC (8), the BNP (7), and the MLC3f (44). EMSAs with nuclear extracts from cardiac cells and with competitors containing either mutated G1 or mutated G2 shows that GATA binding at G1 requires intact G2, whereas binding at G2 occurs even in absence of intact G1, but at a lower rate. An additional GATA element (G3) is located upstream from the transcriptional start site. Intact GATA elements are required for activation of the gene *in vitro* in fetal cultured cardiac cells because mutations that abolish G1/G2 or G3 binding activity also inhibit promoter expression in transfected cardiac cells. A single GATA element, recently identified in the rat cTNI gene promoter, has been shown to activate the gene *in vitro* through interaction with GATA-4 (16). Our study shows that GATA elements in the adult heart are less important than in cultured cardiac cells. This difference could mirror a different level of GATA factors in cardiac nuclei, since we observed that GATA binding activity in adult cardiac cells is much lower compared with fetal cardiac cells. It is possible, therefore, that similarly to MyoD and myogenin in adult skeletal muscle (45), GATA factors undergo down-regulation in terminally differentiated cardiac cells and are no longer required for the maintenance of gene expression. There are conflicting reports on the role of GATA factors in differentiated cardiac cells. In the absence of GATA-4, differentiation of P19 embryonic stem cells is blocked at the cardioblast stage (46, 47). On the other hand, forced expression of GATA-6 in *Xenopus* embryos blocks terminal differentiation of the heart and when exogenous GATA-6 levels drop, the differentiation program is completed (48). One possible interpretation of these data is that different members of the GATA family play different roles in cardiac differentiation. Alternatively, GATA proteins may interact with other regulatory factors to specify their function. The weak role of the cTNI GATA element in the adult heart stands in contrast to that found for the α -MHC gene in which two GATA elements are absolutely required for gene activation *in vivo* (8). One possible explanation for such a discrepancy is that different GATA factors activate the cTNI and α -MHC gene. It is noteworthy, in this respect, that GATA family members differentially transactivate the cTNI promoter in 3T3NIH cells. Particularly, GATA-5 and GATA-6 drive more robust transactivation than GATA-4. GATA-4 and GATA-6 are potent activators of cardiac transcription of many genes, including the α -MHC (8) and the cardiac/slow TnC (10, 49), the BNP and atrial natriuretic factor promoters (7, 50). Despite their structural similarity, however, they are certainly functionally distinct. GATA-6 is unable to substitute for GATA-4 in GATA-4 null mice (3, 4), and GATA-4 but not GATA-6 efficiently interacts and synergizes with Nkx 2.5 (50). The differential ability of GATA-4, GATA-5, and GATA-6 to transactivate the cTNI promoter could be dependent upon binding of GATA proteins with other factors. GATA family members can interact with several transcription factors, and, in the case of the hematopoietic system, cofactors binding to GATA proteins have been demonstrated (51). Protein-protein interaction studies have shown that GATA-4 physically interacts with Nkx 2.5 (50) and NF-AT3 (52) and synergistically activates transcription. It will be interesting to see whether the same proteins or other transcription factors act as GATA cofactors to activate the cTNI gene.

Another major finding of this study is that the proximal cTNI promoter is able to control cardiac specific expression of a reporter gene in transgenic mice. The cTNI transgene shows

early up-regulation during cardiogenesis and strong expression in the adult heart, thus indicating that the transcriptional information within this short sequence is able to control temporal and spatial gene activation. Expression of a promoter in transgenic animals is particularly meaningful, because promoters generally classified as active on the basis of *in vitro* experiments can lead to no or little expression when integrated into the genome. In the biomedical application context, the finding that a small promoter region targets expression of a sequence in the adult heart is of potential importance for gene therapy. Moreover, given its well characterized sequence, the cTNI/*lacZ* transgene may be useful both to investigate whether gene activation in the cardiac chambers requires identical or different combinatorial pathways and to evaluate the importance of other genes in modulating cardiac specific transcription.

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