Divergent transcriptional enhancer factor-1 regulates the cardiac troponin T promoter

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Azakie, Anthony, Lauren Lamont, Jeffrey R. Fineman, and Youping He. Divergent transcriptional enhancer factor-1 regulates the cardiac troponin T promoter. Am J Physiol Cell Physiol 289: C1522–C1534, 2005. First published July 27, 2005; doi:10.1152/ajpcell.00126.2005.—MCAT elements are essential for cardiac gene expression during development. Avian transcriptional enhancer factor-1 (TEF-1) proteins are muscle-enriched and contribute to MCAT binding activities. However, direct activation of MCAT-driven promoters by TEF-1-related proteins has not been uniformly achieved. Divergent TEF (DTEF)-1 is a unique member of the TEF-1 multigene family with abundant transcripts in the heart but not in skeletal muscle. Herein we show that DTEF-1 proteins are highly expressed in the heart. Protein expression is activated at very early stages of chick embryogenesis (Hamburger-Hamilton stage 4, 16–18 h), after which DTEF-1 becomes abundant in the sinus venosus and is expressed in the trabeculated ventricular myocardium and ventricular outflow tracts. By chromatin immunoprecipitation, DTEF-1 interacts with the cardiac troponin T (cTnT) promoter in vivo. DTEF-1 also interacts with MEF-2 by coimmunoprecipitation and independently or cooperatively (with MEF-2) trans-activates the cTnT promoter. DTEF-1 isoforms do not activate the cTnT promoter in fibroblasts or skeletal muscle. DTEF-1 expression occurs very early in chick embryogenesis (16–18 h), preceding sarcomeric protein expression, and it activates cardiac promoters. As such, DTEF-1 may be an early marker of the myocardial phenotype. DTEF-1 trans-activates the cTnT promoter in a tissue-specific fashion independent of AT-rich, MEF-2, or GATA sites. The observed spatial pattern suggests decreasing levels of expression from the cardiac inlet to the ventricular outflow tracts, which may mark a cardiogenic or differentiation pathway that parallels the direction of flow through the developing chick heart.

cardiac differentiation; MCAT sites; monocyte enhancer factor-2; cell specification

NUMEROUS CARDIAC GENES, including the α- and β-myosin heavy chain (MHC), myosin light chain, and the cardiac troponin T (cTnT), C, and I genes, share common regulatory promoter motifs and are simultaneously expressed during myocardial differentiation (3, 11–13, 28–30, 35). To study the transcriptional mechanisms that operate during myogenesis, we use the avian cTnT gene as a model of cardiac gene expression (21–24). The avian cTnT gene is expressed in embryonic cardiac and skeletal muscle and is restricted to cardiac muscle later in development (22). The cTnT gene contains a TATA box, a GC box, and MCAT motifs within the proximal promoter (24). An upstream “cardiac element,” with putative monocyte enhancer factor-2 (MEF-2), MEF-3, and GATA elements is necessary for full promoter activity in cardiac myocytes but is dispensable for activity in skeletal myocytes (14, 23).

MCAT elements govern muscle-specific gene expression. The MCAT motif (CATTCCT) is present in two copies in the cTnT gene promoter, both of which are important for cardiac and skeletal muscle gene activation in vitro (21–24) and in vivo (36). MCAT motifs are necessary for the activity of other cardiac promoters, including skeletal α-actin, β-MHC, α-MHC, troponin C, and myosin light chain (11–13, 17, 28–30). MCAT elements are also present in nonmuscle promoters, including those of the human papillomavirus, simian virus (SV)40, and somatomammotropin genes (15, 31, 32, 34, 38). How MCAT elements control muscle-specific expression and mediate different effects in nonmuscle tissues is not entirely clear. The flanking sequences of MCAT elements have been shown to modulate the specificity of muscle and nonmuscle transcription (17), and MCAT binding factors may require tissue-specific coactivators or corepressors for transcriptional activation (4).

Avian TEF-1 proteins, which are muscle-enriched, contribute to MCAT binding activities in vitro and in vivo. We have previously shown (2) that vertebrate TEF-1 belongs to a multigene family of at least four members: nominal (N)TEF-1, related (R)TEF-1, divergent (D)TEF-1, and embryonic (E)TEF-1. Messenger RNA from two TEF-1-related genes show distinct tissue distribution, one enriched in skeletal and cardiac muscle (RTF-1) and the other enriched in cardiac muscle only (DTEF-1). NTEF-1 shows highest homology with TEF-1 cloned from HeLa cells, which binds to the Sp1 and GTIIC motifs (the sequences of which are closely related to the MCAT motif) of the SV40 enhancer (8, 38). Despite the presence of endogenous TEF-1 within HeLa cells, transfection of exogenous human TEF-1 into HeLa cells does not increase activity of GTIIC-driven reporter genes. In myocyte culture systems, exogenous RTEF-1 did not trans-activate the cTnT promoter (33). In addition, exogenous TEF-1 was not active in cell lines lacking endogenous TEF1-related proteins. These observations have led to the belief that cofactors or coactivators may be necessary for tissue-specific gene activation, and/or that TEF-1 belongs to a multigene family, whose members have both tissue-specific and redundant functions. The identification of tissue-specific cofactors or coactivators remains to be completely elucidated, but it is apparent that polyl(ADP-ribose) polymerase, vestigial-like gene products and MEF-2 possibly interact with TEF-1 to regulate muscle-specific transcription (4, 18, 19).

DTEF-1 is a unique member of the TEF-1 multigene family with highly abundant transcripts in heart but not skeletal muscle (2). The ability of DTEF-1 to trans-activate cardiac transcription...
promoters in primary avian cardiac cell culture is not known. Murine DTEF-1 (TEF-5) is important in mediating the effects of α-adrenergic stimulation on muscle gene expression (20). Murine DTEF-1, however, has no effect on MLC2v promoter activity and only mild activation of β-MHC promoter (19) in cotransfection experiments in CV-1 cells (African green monkey kidney cells). The MEF-2-dependent activation of the MLC2v and β-MHC promoters in CV-1 cells is squelched by TEF-1 factors. Herein we examine the protein expression patterns of DTEF-1 and its contribution to MCAT binding activities in embryonic chick tissues in vitro and in vivo. We test the hypothesis that DTEF-1 independently and directly trans-activates the cTnT gene and show that its transactivating function is tissue-specific and independent of upstream AT-rich/MEF-2 and/or GATA promoter motifs.

MATERIALS AND METHODS

Reagents. Oligonucleotides were purchased from Operon Technologies (Alameda, CA) or Invitrogen (Carlsbad, CA). Media, serum, enzymes, reagents, and materials for tissue culture were purchased from the Cell Culture Facility at the University of California, San Francisco.

Plasmid constructs. The promoter and reporter constructs used in this study consist of 129 and 268 nucleotides of the chicken cTnT promoter upstream of the transcription initiation site, respectively. These promoter constructs were originally generated by nested deletions of the chicken cTnT gene (7) in Bluescript KS. These promoter constructs were created by digestion of the above promoter inserts with XhoI and Xhol restriction sites of the eukaryotic expression vector pcDNA4/His Max-A (Invitrogen). All constructs were confirmed using standard sequencing methods.

Nuclear extracts. All tissues (cardiac muscle, skeletal muscle, fibroblasts, lung, liver, kidney, brain, and gizzard) were harvested from embryonic day 12 chicks at 4°C. Muscle tissues were placed in relaxation buffer, rinsed in homogenization buffer, and mechanically homogenized. The nuclei were then pelleted and resuspended in lysis buffer. After the protein concentration was determined with the use of the Bradford method, the nuclear extracts were snap-frozen and stored at −80°C (2, 9, 10, 14, 17, 21–24, 33).

Protein expression. Full-length chicken D-TEF1A/B or R-TEF1A cDNA in frame in pRSET-A expression vector (Invitrogen) was used for protein expression. BL21(DE3)pLysS competent cells were transformed with the expression construct and plated according to the manufacturer’s protocols (pRSETA, B, and C, for high-level expression of recombinant proteins in Escherichia coli v351–20; Invitrogen). Expressed proteins were purified using the Probound purification system (Invitrogen). Mobility shift assays, competition, and supershift assays. Gel shift probes were made from 23-mer oligonucleotide fragments of MCAT-1 (sense strand sequence: TGGCAAGTTGTCATTCTCCTTCG and MCAT-2 (TGGCCCGGGCAACTCTCCTTCG) as previously described (17). Mutant competitor had the following sequences: MCAT-1 mutant, 5′-TGGCAAGTTGTCATTCCCTTCG-3′; MCAT-2 mutant, 5′-TGGCCCGGGCAACTCTCCTTCG-3′. Supershift assays were performed using monoclonal TEF-1 antibody (BD Transduction Laboratories) and antisera specific to DTEF-1 (AnaSpec, San Jose, CA). DTEF-1 rabbit antisera was made to immunogen peptide, which corresponds to amino acid residues 138–154 (SVLQNKLSPPPLPQAV) of chick DTEF-1.

Western blot analysis. Total protein concentration in the samples were quantitated with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Parallel blots probed for histone H1 and nucleoprotein were performed to verify sample integrity (data not shown). An equal amount of protein was loaded in each lane for Western blot analysis. SDS-PAGE was used to separate nuclear proteins (10 µg) on a 10% gel, followed by transfer to a polyvinylidene difluoride membrane. The membranes were then blocked with 5% nonfat dried milk in 130 mM NaCl and 25 mM Tris (pH 7.5) for 1 h at room temperature. After being washed in Tris-buffered saline and 0.05% Tween 20 (TBST), the membranes were incubated in antibody (0.5–2.0 µg/ml IgG). The immunoblot was incubated at 4°C overnight and then washed in TBST. Goat anti-rabbit IgG peroxidase in TBST-2% goat serum was then added to the incubation for 1 h at 4°C, followed by washing in TBST. Chemiluminescence was then used for visualization of bands.

To identify proteins in gel shift complexes (10), the mobility shift reactions were scaled up fivefold in a final volume of 20 µl. Following electrophoresis, the gels were soaked in 2% SDS, 62.5 mM Tris, 25 mM dithiothreitol, and then dried. The DNA-protein complexes were then identified by autoradiography, and the band of interest was excised and loaded on a 10% SDS-PAGE gel. The immunoblot analysis was then carried out as described above.

Chromatin immunoprecipitation. All chromatin immunoprecipitation (ChIP) assays were performed based on the protocols of the chromatin immunoprecipitation assay kit (Upstate, Lake Placid, NY). Primary antibody (5 µg) against DTEF-1, MEF-2, and GATA-4 was added to a 500-µl chromatin sample for the IP reaction. ChIP dilution buffer and preimmune rabbit serum were used as controls for nonspecific interactions and DNA contamination. Anti-β-actin and anti-cTnT antibodies were used as negative controls.

PCR. After DNA purification, samples were subjected to PCR with primers designed for the chick heart cTnT promoter (cTnT-268) as follows: upper primer, 5′-GCTGGCTGCTTGTTGTCGA-3′, and lower primer, 5′-CTTGGGCGGACAGGCTT-3′. The primers used for PCR were designed using primer analysis software (Oligo 6.8, Molecular Biology Insights, Cascade, CO). The amplified PCR product is 265 bp.

Commmunoprecipitation. Preparation of the immune complexes was performed using the Seize classic (A) immunoprecipitation kit (Pierce Biotechnology, Rockford, IL). Nuclear extracts or whole cell lysates were incubated with 5 µl of anti-DTEF-1 antisera or control IgG or preimmune serum or no antibody/antiserum in binding buffer overnight at 4°C. Membranes were probed separately with TEF-1 antibody (BD Transduction Laboratories), anti-DTEF-1 antisera (AnaSpec), MEF-2 antibody (Santa Cruz Biotechnology), and preimmune antisera (AnaSpec).

Tissue culture, cell transfections, and reporter gene assays. All tissues (cardiac muscle, skeletal muscle, and fibroblasts) were harvested from embryonic days 6–12 chicks using standard techniques (2, 9, 10, 14, 17, 21–24, 33). Differentiation states were verified by cell fusion (light microscopy), Western blots, and immunohistochemistry for cell-specific proteins (MEF-2, myosin, cTNT, Nkx2.5, actin, and vimentin; data not shown). Plasmid constructs were transfected according to the manufacturer’s protocol (Effectene Transfection kit; Qiagen) with minor modifications. Briefly, cultured cells were washed and resuspended in serum-free medium. −129CtvtTGGlL2 or −268CtvtTGGlL2 wild-type and mutated (MCAT-1, MCAT-2, or both sites) promoter constructs (300 ng) were transfected. MEF-2 (gift from Dr. B. Black) or DTEF-1A or B expression constructs (pcDNA4/His Max; Invitrogen) were cotransfected at concentrations ranging from 2 to 2,000 ng/200 µl. Reporter gene activity was determined using the Luciferase reporter assay (Promega). Firefly light intensity was read on a luminometer TD-20/20, DLR Ready (Turner Designs Instrument, Sunnyvale, CA). Cotransfection with TK reporter was used to standardize for transfection variability. Cotransfection of DTEF-1 expression constructs was verified using Western blot analysis of both His-tagged DTEF products and probing blots with DTEF antibody.
Cotransfection with GFP-expressing constructs was performed to verify efficiency (skeletal muscle 69%, cardiac muscle 71%, and fibroblasts 75%) as follows: The cells were transfected with mammalian fluorescent protein vector pAAV-Hr-GFP (Stratagene, La Jolla, CA) according to the manufacturer’s protocol (Effectene Transfection kit; Qiagen). The GFP-positive cells were viewed with the use of a Zeiss fluorescence microscope with a green fluorescence filter. The transfection efficiency was calculated as the GFP-positive cells/total cell counted ratio and expressed as a percentage. Luciferase reporter gene activity is expressed as means ± SD. Statistical comparisons were performed using paired t-tests, with statistical significance set at 0.01.

**Embryo immunostaining.** Embryos for sectioning were harvested between Hamburger and Hamilton (HH) stage 16 (51–56 h) and HH stage 18 (65–69 h). Once harvested, the embryos were fixed overnight in PBS containing 4% paraformaldehyde at 4°C and then washed for 2 to 3 h in PBS in 15% sucrose-PBS overnight. Embryos were then embedded in Tissue-Tek optimal cutting temperature compound and frozen in 2-methylbutane cooled by a liquid nitrogen bath. Ten-micrometers-thick sagittal sections and cross-sections were collected.

**Fig. 1.** A: Western blot analysis using anti-serum against divergent transcriptional enhancer factor-1 (DTEF-1) peptide (SVLQNKLSPPPPLPQAV). The DTEF-1 antiserum specifically recognizes expressed RSET-DTEF-1 protein, but not expressed RSET-RTEF-1. MW, molecular weight. B: TEF-1 monoclonal antibody identifies both bacterially expressed RTEF-1 and DTEF-1 products. TEF-1 proteins are widely expressed in embryonic chick tissues. H, Heart; SM, skeletal muscle; F, fibroblasts (skin); Lg, lung; K, kidney; GI, gastrointestinal tract; GZ, gizzard; DTEF, bacterially expressed RSET-DTEF protein; RTEF, bacterially expressed RSET-RTEF protein. C: DTEF-1 protein is highly enriched in embryonic chick heart, but not in skeletal muscle. Low levels were observed in kidney and gut. With longer exposures, trace amounts of protein were observed in the lung and gizzard. DTEF-1 products in heart might include 52-, 54-, and 57-kDa polypeptides (see Fig. 3, Ref. 10). D: DTEF-1 protein is expressed as early as 16–18 h in the embryonic chick. DTEF-1 expression precedes that of cardiac troponin T (cTnT) protein products. E1–E6 = whole chick embryo extracts; E8–E15 = embryonic heart nuclear extracts.
with a Leica CM1900 cryostat on Super Frost slides (Fisher). The slides were subsequently processed for immunohistochemistry. Slides were first air dried at room temperature then postfixed in 0.2% PFA in PBS for 10 min, followed by three 5-min washes in 1× PBS to remove the optimum cutting temperature compound. After being washed, slides were blocked for 30 min in blocking solution consisting of 1% BSA and 10% goat serum in PBS. Anti-DTEF antibody was diluted 1:200 in blocking solution, and the slides were incubated for 2 h in a humidified slide box. After primary antibody staining, the slides were washed twice in 1× PBS, followed by a wash in 4× PBS. The anti-DTEF antibody was visualized using goat anti-rabbit Alexa Fluor 488 (Molecular Probes) diluted 1:500 in blocking solution and allowed to incubate for ~1 h in a slide box. Slides were mounted with glass coverslips and Biomedia Gel Mount and viewed with a Zeiss Axioskop 2 with AttoArc 2 Variable Intensity HB100 Arc Lamp.

**RESULTS**

**DTEF-1-specific antiserum.** The derived amino acid sequence of DTEF-1 shows that the region spanning amino acids 138–154 (20) has a low sequence similarity with other TEF-1 family members (NTEF-1 and RTEF-1). An epitope/immuno-
DTEF-1 polypeptide but not RTEF-1 (Fig. 1B). The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to polyvinylidenedifluoride membrane, and probed with MEF-2 antibody (Ab) (A), DTEF-1 antiserum (B), preimmune serum (C), or monoclonal TEF-1 antibody (D). Each panel shows the following: lane 1, lysate of primary cardiomyocyte cultures; lane 2, lysate of primary cardiomyocyte cultures cotransfected with DTEF-1 (pcDNA4HIS-DTEF-1); lane 3, DTEF-1 Ab immunoprecipitate of embryonic heart nuclear protein extract; lane 4, DTEF-1 Ab immunoprecipitate of cultured embryonic cardiac myocyte lysate; lane 5, preimmune serum immunoprecipitate of embryonic heart nuclear protein extract; lane 6, preimmune serum immunoprecipitate of cultured embryonic cardiac myocyte lysate; lane 7, buffer; lane 8, rabbit IgG control plus immobilized protein A beads; and lane 9, purified bacterially expressed RSET-DTEF-1 protein.

Temporal expression of DTEF-1 in embryonic chick. To determine the onset of expression of DTEF-1 proteins, Western blot analysis of embryonic D1–D8 tissues was performed (HH stages 4–33). Because of the limited amount of tissue available, whole embryo extracts were used for SDS-PAGE separation of embryonic days 1–6 tissues. Once the embryo reached approximately embryonic day 8, selective dissection and pooling of hearts was performed. (Fig. 1D; embryonic days 1–6 = whole embryo extracts and embryonic days 8–15 = heart nuclear extracts).

DTEF-1 is expressed as early as 16–18 h of chick embryogenesis. From embryonic day 1 through day 15, a single isoform was detected using Western blot analysis. The onset of expression of DTEF-1 proteins preceded that of cTnT protein products, which were detected by 48–72 h of chick embryogenesis.

DTEF-1 contributes to all MCAT binding factor mobility shift complexes in embryonic cardiomyocytes. Gel retardation and supershift assays were performed using MCAT-1 and MCAT-2 23-mer probes, as well as DTEF-1 antiserum and monoclonal TEF-1 antibody to determine the presence and contribution of DTEF-1 proteins to MCAT binding activities in the embryonic avian myocardium. The complexes resulting from the binding reaction using MCAT-1 DNA are shown in Fig. 2A. Three mobility shift complexes (lane 1) are shown (C1, C2, and C3), consistent with previous detailed descrip-
tions (10), and are produced by TEF-1 protein interactions with the core MCAT motif (17). All three complexes were competed when 200-fold excess of unlabeled MCAT-1 DNA (lane 3) was added to the binding reaction. There was no competition of complexes when mutant MCAT-1 oligonucleotide (lane 4) was used, indicating that the MCAT-1 DNA protein binding is sequence specific. The addition of DTEF-1-specific antiserum produced a supershifted complex with much slower mobility (lane 5). All three complexes were supershifted by the DTEF-1 antiserum, suggesting that DTEF-1 proteins contributed to MCAT-binding activities in embryonic avian myocardium. The addition of preimmune serum did not supershift any of the three mobility shift complexes (lane 6).

When MCAT-2 oligonucleotide was used as a probe, three similar mobility shift complexes were produced (Fig. 2B, lane 2: C1, C2, and C3). The binding of MCAT-2 DNA to TEF proteins in cardiac nuclear extract was also sequence-specific because addition of excess unlabeled MCAT-2 oligonucleotide (lane 3) competed for protein binding and mutant DNA competitor (lane 4) did not. The addition of DTEF-1 antiserum produces a supershift of all three complexes (lane 5), suggesting that DTEF-1 proteins contributed to each DNA protein complex.

MCAT-1 and MCAT-2 binding complexes (C1, C2, and C3) are composed of different DTEF-1 polypeptides. Elution of proteins from mobility shift complexes has shown that all MCAT binding activities contain TEF-1 proteins (10). Conversely, a direct comparison of Western and Southwestern blots of embryonic chick tissues has shown that proteins that bind MCAT DNA are TEF-1 related. We sought to determine the contribution of DTEF-1 proteins to MCAT binding activities in embryonic chick heart using both MCAT-1 and MCAT-2 23-mer probes. DNA binding reactions between cardiac nuclear protein and MCAT-1 or MCAT-2 23-mer probes were run on high-resolution mobility shift assays. Western blot analysis of proteins eluted from complexes C1–C3 derived from embryonic chick heart was performed using DTEF-1-specific antibody.

Western blot analysis of proteins eluted from all three complexes derived from either MCAT-1 or MCAT-2 probe shows that three DTEF-1 polypeptides contribute to MCBF activities (Fig. 3). The three polypeptides are comparable to the 52-, 54-, and 57-kDa proteins identified in previous Western blot analyses of proteins eluted from MCBF complexes described by Farrance et al. (10).

**DTEF-1 interacts with MEF-2 factors detected by coimmunoprecipitation.** TEF-1 factors have been shown to interact with MEF-2 factors and potentially interfere with MEF-2 dependent activation of muscle-specific genes in noncardiac cells (19). To determine whether DTEF-1 specifically interacts with MEF-2 in embryonic chick heart, cardiac nuclear extracts, or whole cell lysates were incubated with DTEF-1 antiserum, control IgG, preimmune serum, or no antibody/antiserum. The protein complexes were eluted from protein A beads, separated by SDS-PAGE, and transferred to a membrane, which was probed separately with DTEF-1 antisera, preimmune anti-
serum, and MEF-2 antibody and TEF-1 antibody. The DTEF-1-immunoprecipitated protein complex contained MEF-2 (Fig. 4A, lanes 3 and 4) and DTEF-1 (Fig. 4B, lanes 3 and 4). Control IgG or preimmune serum immunoprecipitates did not contain DTEF-1 (Fig. 4B, lanes 5, 6, and 8) or MEF-2 (Fig. 4A, lanes 5, 6, and 8). Western blot analysis of DTEF-1 immunoprecipitates from cardiomyocyte culture lysates (Fig. 4C, lanes 1 and 2) and immunoprecipitates of cardiomyocyte nuclear extracts (Fig. 4C, lanes 3 and 4) probed with preimmune serum showed no protein bands. A similar blot probed with TEF-1 monoclonal antibody (Fig. 4D) showed only TEF-1 protein in immunoprecipitates from nuclear extracts (Fig. 4D, lanes 3 and 4). No TEF-1 band was observed in DTEF-1 immunoprecipitates from cell lysates (Fig. 4D, lanes 1 and 2), probably because the overall nuclear protein concentration was low relative to immunoprecipitates derived from cardiac nuclear extracts.

Lysates of cultured embryonic cardiomyocytes contained DTEF-1 protein that we observed using Western blot assay (Fig. 4B, lane 1). Transfection of cultured embryonic cardiomyocytes with expression constructs encoding DTEF-1 showed an overexpression of DTEF-1 (Fig. 4B, lane 2) within the cardiac cells, suggesting that the protein is expressed upon transfection.

**DTEF-1A and DTEF-1B isoforms do not trans-activate the cTnT promoter in embryonic skeletal myocytes or fibroblasts.** High levels of cTnT promoter activity in embryonic chick cardiomyocytes required a promoter construct (−268cTnT) that contained putative MEF-2/AT-rich MEF-3 and GATA sites (Fig. 5B). The −129cTnT promoter (−129cTnT), which contained tandem MCAT sites, had −12–20% activity relative to −268cTnT promoter in embryonic cardiomyocytes (Fig. 5A). To determine the tissue specificity of the trans-activating function of DTEF-1A or -1B on the cTnT promoter, cotransfection experiments were performed using primary cultures of embryonic fibroblasts and skeletal myocytes. Cultured embryonic skeletal myocytes or fibroblasts were transiently transfected with −129cTnT or −268cTnT promoter constructs upstream of a luciferase reporter gene, and DTEF-1A or DTEF-1B expression constructs. The concentration of DTEF-1 expression construct that was used for transfection ranged from 2 to 2,000 ng/200 μl (Fig. 6A) DTEF-1A or -1B did not trans-activate reporter gene expression under −268cTnT or −129cTnT promoter control in primary cultures of embryonic fibroblasts or skeletal myocytes (Fig. 6B) compared with baseline.

**DTEF-1A and DTEF-1B isoforms trans-activate the −129cTnT promoter in embryonic cardiomyocytes independent of the upstream “cardiac element.”** Because the −129cTnT promoter contains MCAT sites, the DNA binding motif for TEF-1 factors, we sought to determine the trans-activating effects of DTEF-1A and -1B on the −129cTnT promoter in cardiac myocytes. Although the −129cTnT promoter has relatively low activity in embryonic cardiomyocytes (compared with −268cTnT), cotransfection of DTEF-1B or -1A in cardiomyocytes resulted in an increase in −129cTnT promoter activity. DTEF-1B trans-activated (Fig. 7A) the −129cTnT promoter 1.8-fold compared with baseline (476 ± 204 units, n = 20 vs. 262 ± 157 units, n = 18; P < 0.01). DTEF-1A resulted in a 3.5-fold increase in −129cTnT promoter activity (Fig. 7B) compared with baseline (1,034 ± 89 vs. 290 ± 11 units, n = 3 each; P < 0.01). To determine whether the effect was tissue specific, reporter gene constructs under −129cTnT promoter control were cotransfected with DTEF-1A or -1B expression constructs into primary embryonic skeletal myocytes or fibroblasts. DTEF-1A or -1B isoforms did not trans-activate the −129cTnT promoter in primary cultures of embryonic fibroblasts or skeletal myocytes compared with baseline (Fig. 6B).

**Both DTEF-1A and DTEF-1B isoforms trans-activate the −268cTnT promoter in embryonic cardiomyocytes.** To determine the ability of DTEF-1 isoforms to trans-activate the −268cTnT promoter in embryonic chick cardiomyocytes, expression constructs of each isoform were cotransfected at increasing concentrations (2–1,000 ng/200-μl well). Cotransfection of DTEF-1B with the −268cTnT promoter construct results in a 2.3-fold trans-activation (Fig. 7A) compared with baseline (−268cTnT, 2,082 ± 965 units; n = 18 vs. −268cTnT + DTEF-1B 4,870 ± 2,110 units; n = 20, P < 0.01). The trans-activation was dose dependent and optimal at 2–100 ng of cotransfected DTEF-1B expression construct (data not shown). Cotransfection of DTEF-1A expression vector with −268cTnT promoter constructs (Fig. 7B) resulted in a 2.2-fold increase in promoter activity compared with baseline (−268cTnT, 2,220 ± 88 units; n = 3 vs. −268cTnT + DTEF-1A 4,800 ± 403 units; n = 3; P < 0.01). The trans-activating response was also dose dependent. Maximal cTnT promoter trans-activation was achieved by cotransfection of 2–20 ng of DTEF-1A expression construct (data not shown). The degree of −129cTnT promoter trans-activation by
DTEF-1A or DTEF-1B is comparable to that shown for the 
268cTnT promoter.

**MCAT sites are essential for DTEF-1 activation of the cTnT promoter.** To determine whether the trans-activating function of DTEF-1 on the cTnT promoter was nonspecific or dependent on functional DTEF-1 DNA binding sites, MCAT sites of the −268cTnT promoter construct were mutated and used in cotransfection experiments (Fig. 8). Reporter gene activity is significantly reduced when DTEF-1 binding is abrogated by mutations in either the MCAT-1 or the MCAT-2 site or both in the context of the −268cTnT promoter.

−268cTnT promoter activity (Fig. 8, A and B, lane 1) was increased >200% above baseline by DTEF-1A or DTEF-1B (Fig. 8, A and B, lane 2) cotransfection in cultured embryonic cardiac myocytes. Mutations in either MCAT-1 or MCAT-2 sites or both resulted in a reduction of −268cTnT promoter activity (Fig. 8, A and B, lane 2).Reporter gene activity was increased by 100% above baseline by DTEF-1A or DTEF-1B cotransfection in cultured embryonic cardiac myocytes. Mutations in either MCAT-1 or MCAT-2 sites or both resulted in a reduction of −268cTnT promoter activity (Fig. 8, A and B, lane 2).
DTEF-1A and DTEF-1B interact with MEF-2C to trans-activate the cTnT promoter. The functional implications of a DTEF-1-MEF-2 interaction by communoprecipitation (Fig. 4) were investigated by cotransfections of DTEF-1A and -1B and MEF-2C expression constructs with −268cTnT promoter driving a luciferase reporter gene. Cotransfection of MEF-2C with −268cTnT promoter increased reporter gene activity 3.4-fold compared with baseline (Fig. 9, lane 4). Cotransfection of DTEF-1A or DTEF-1B and MEF-2C with −268cTnT promoter resulted in a 4.2- or 5.4-fold increase, respectively, in baseline reporter gene activity (P < 0.001) (Fig. 9, lanes 5 and 6).

DTEF-1 binds cTnT promoter as detected by chromatin immunoprecipitation. Having shown that DTEF-1 contributes to MCAT binding activities in the heart, binds MCAT sites in a sequence-specific fashion, and trans-activates the cTnT promoter, we sought to determine whether DTEF-1 binds the cTnT promoter in vivo by chromatin immunoprecipitation. After cross-linking and shearing, chromatin from embryonic chick tissues was immunoprecipitated with DTEF-1 antibody, GATA-4 antibody, MEF-2 antibody, preimmune serum, anti-cTnT antibody, and anti-β-actin antibody. The immunoprecipitated complexes were then eluted, and protein-DNA cross-links were reversed. DNA from the immunoprecipitated sample was then purified and subjected to PCR with primers designed specifically for the chick cTnT promoter (proximal 265 bases). Antibody to DTEF-1, MEF-2, or GATA factors immunoprecipitates chromatin from cardiac nuclear extracts that produces a PCR fragment spanning the 265-bp region upstream of the transcription initiation site of the cTnT promoter region, implying that these factors bind the promoter in

activity to ~20% (Fig. 8, A and B, lanes 3–5) of baseline, despite the upstream AT-rich, MEF-2, and GATA sites. Furthermore, neither DTEF-1A (Fig. 8A, lanes 6–8) nor DTEF-1B (Fig. 8B, lanes 6–8) cotransfections with mutated promoters (−268cTnT with mutated MCAT-1 or MCAT-2 sites) could produce any increase in mutant promoter activity.
vivo. Antibody to cTnT, β-actin, or preimmune serum does not immunoprecipitate chromatin that yields a comparable PCR fragment. Figure 10 shows that the cTnT promoter, within the context of chromatin in vivo, is bound by GATA, MEF-2, and DTEF-1 factors in cardiac nuclear extracts.

DTEF-1 is differentially expressed in developing segments of chick heart. Immunostaining of HH stages 16–18 (51–69 h, approximately embryonic day 3) chick embryos showed that DTEF-1 is relatively strongly expressed in the inflow region of the developing heart, near the atrioventricular groove or fold, as well as in the sinus venosus (Fig. 11). The trabeculated myocardium of the looping ventricle showed relatively lower levels of DTEF-1 expression. By HH stage 18, DTEF-1 was also expressed in the outflow tracts, but at lower levels compared with the cardiac inlet (Fig. 11C). Parallel staining of embryos with DAPI suggested perinuclear localization of DTEF-1 expression in cardiocytes in the cardiac inlet and ventricular myocardium. Furthermore, double staining with MF-20 (and HV-11) suggested that DTEF-1 colocalizes within cardiac myocytes (data not shown).

**DISCUSSION**

The TEF-1 family is a member of the TEA/ATTS domain family of transcription factors, which is conserved across many species and plays an important role in retrotransposon activation (TEC1 in yeast), conidiation (ABAA in Aspergillus nidulans), and neurodifferentiation (scalloped in Drosophila) (1, 5, 16, 27). TEF-1 multigene family members function in the 2- to 8-day cell stage murine embryo (25), in which knockout produces embryonic lethality due to myocardial hypoplasia (6). In mammals and other vertebrates, TEF-1 factors contribute to MCAT binding activities to regulate muscle- and non-muscle-specific gene expression. The mechanism(s) by which TEF-1 factors regulate tissue-specific gene expression are poorly understood. Human TEF-1, originally cloned in HeLa cells, avidly binds the GT-IIC and Sph enhancers (38). Transfection of this cloned TEF-1 does not activate these enhancers in cells lacking endogenous TEF-1, but does result in repression of the endogenous TEF-1 activity present in HeLa cells. In chimeras in which the DNA-binding domain of the GAL-4 activator replaces that of TEF-1, transcription in HeLa cells is stimulated, but this phenomenon does not occur in MPC11 cells (where endogenous TEF-1 is lacking), which suggests that the trans-activation function of TEF-1 is mediated by limiting, possibly cell-specific, transcriptional cofactors or coactivators. In keratinocytes, activation of human papillomavirus-16 oncogenes by TEF-1 requires a cell-specific coactivator.

Analysis of the activation of MCAT-dependent muscle promoters by TEF-1 has also been limited. Direct trans-activation of the cTnT gene by RTEF-1 isoforms could not be demonstrated (33), because transfected TEF-1 expression constructs in skeletal myocytes squelch the promoter. To show the activating function of avian RTEF-1 isoforms the TEA domain in cardiac-specific promoters. DTEF-1 consists of two isoforms, A and B, and its RNA expression pattern suggests cardiac but not skeletal muscle enrichment, implicating a role for DTEF 1 in cardiac-specific MCAT-dependent gene expression. Murine DTEF-1 (mTEF-5) is activated during α₁-adrenergic stimulation of cardiac myocytes, potentially contributing to pathophysiological responses of the myocardium to states of increased afterload (20). In other cotransfection experiments, MLCl2v promoters could not be directly trans-activated by DTEF-1, and β-MHC promoters were only mildly activated, in CV-1 cells (19). Herein we report that avian DTEF-1 directly trans-activated reporter gene driven by cTnT promoter constructs in primary cultures of embryonic cardiomyo-

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**Table 1**

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**Fig. 10. Chromatin immunoprecipitation (ChIP).** PCR using primers specific for cTnT promoter on DNA purified after ChIP. Technical details are described in MATERIALS AND METHODS. After being cross-linked and sonicated, chromatin complexes from embryonic chick nuclear preparations were purified. After being heated at 65°C, the input chromatin was incubated with antibodies to DTEF-1, GATA, β-actin, cTnT, preimmune serum, and MEF-2. Separation of immune complexes by and elution from protein A/G agarose beads was followed by reversal of protein-DNA cross-links and DNA purification. Lane 1 shows chromatin input DNA plus antibody. Lanes 1–5 include the ChIP “output” DNA for PCR reactions. The immunoprecipitation of chromatin preparations was performed with antibodies listed at left. Lane 6 contains ChIP buffer as the DNA source. Lane 7 contains the control, “input DNA” to verify the quality of nuclear preparations.
ocytes. Cotransfection of reporter gene constructs under cTnT promoter control with DTEF-1 expression constructs produced increases in cTnT promoter activity. Unlike RTEF-1, in which differential activation between isoforms was shown with GAL-4-DBD fusion constructs, both DTEF-1A and DTEF-1B directly trans-activated the cTnT promoter. The trans-activating function of avian DTEF-1 is dose dependent, but very high concentrations seemed to interfere with reporter gene activity.

The dose-dependent nature of the trans-activating function of DTEF-1 may suggest that previous failed efforts to show direct TEF-1 activation of MCAT-dependent promoters may have been due to the use of relatively high concentrations of cotransfected TEF-1 expression constructs in those experiments. The higher concentrations may prove to be toxic to cells or may squelch or interfere with reporter gene constructs. If this were the case, then lower concentrations would also activate MCAT-dependent promoters in other cells, including skeletal myocytes or fibroblasts. However, even variable concentrations of cotransfected DTEF-1 do not trans-activate the cTnT promoter in skeletal myocytes or fibroblasts. The trans-activating function of DTEF-1 seems to be unique to it, compared with NTEF and RTEF-1, and it is tissue-specific and restricted to cardiac muscle, arguing for the presence of cardiac-specific coactivators.

Because scalloped interacts with vestigial to control wing differentiation in Drosophila, TEF-1 family members also interact with vestige-like products in skeletal muscle (18, 19, 26, 37). Vestige-like products are absent in cardiac muscle, but TEF-1 family members might also interact with MEF-2 and/or poly(ADP-ribose) polymerase to potentially regulate tissue-specific transcription (4, 19). TEF-1 factors differentially modulate MEF-2-dependent activation of MLC2v and β-MHC.

Fig. 11. Expression patterns of DTEF-1 in the embryonic chick. A: cross-section of Hamburger and Hamilton (HH) stages 16–18 chick embryo showing immunostaining with anti-DTEF-1 antibody. DTEF-1 expression is marked in the inflow region of the heart. Mild staining is shown in the ventricular wall. B: magnification of the ventricle shown in C. DTEF-1 was expressed in the trabeculated portion of the looping ventricle. C: longitudinal/sagittal section through HH stage 18 chick embryo labeled with anti-DTEF-1 antibody. The section demonstrates DTEF-1 expression in the inflow of the heart, near the atrioventricular groove, as well as the outflow.
promoters in transient transfections of cultured CV-1 cells. The activation of these promoters by MEF-2 is squelched by cotransfection of TEF-1 factors. Herein we have shown that DTEF-1 interacts with MEF-2 as detected by coimmunoprecipitation. Furthermore, both factors bind the promoter within the context of chromatin in vivo. Both DTEF-1 and MEF-2 cooperatively activate the −268cTnT promoter construct to produce reporter gene levels higher than those observed for either DTEF-1 or MEF-2 alone. DTEF-1 can directly trans-activate the cTnT promoter in the absence of “required” upstream cardiac element motifs. The ability of DTEF-1 to trans-activate the cTnT promoter independent of upstream MEF-2, GATA-4, and MEF-3 motifs, however, is restricted to cardiac muscle and does not occur in embryonic skeletal myocytes or fibroblasts.

Numerous avian TEF-1 gene products contribute to MCAT binding activities in muscle and nonmuscle tissues (9, 10). Elution of proteins from mobility shift complexes has shown that all MCAT binding activities contain TEF-1 proteins. Conversely, a direct comparison of Western and Southwestern analyses of embryonic chick tissues has shown that proteins that bind MCAT DNA are TEF-1 related. The present study shows that DTEF-1 contributes to MCAT binding activities in heart and binds the cTnT promoter in vivo in the context of chromatin.

TEF-1-related proteins are diverse, consisting of 52-, 54-, and 57-kDa polypeptides (10). All three polypeptides are present in muscle and nonmuscle tissues at various levels and different stoichiometries. The 54-kDa polypeptide is enriched in muscle tissues (cardiac, skeletal, and smooth). The 54- and 57-kDa proteins are phosphorylated, whereas the 52-kDa protein is not, implicating the former two polypeptides in the α-adrenergic response in cultured cardiac muscle (20). Proteolytic digestion mapping of these three polypeptides shows that the 52- and 57-kDa proteins have identical proteolytic cleavage patterns, whereas the 54-kDa polypeptide has a distinct proteolytic map (10). In the present study, we have shown that DTEF-1 products include three polypeptides comparable to the 52-, 54-, and 57-kDa products described by Farrance et al. (10). The three polypeptides can be explained by two observations. First, at least two isoforms of DTEF-1 exist due to alternative splicing of DTEF-1 mRNA, and second TEF-1 proteins undergo posttranslational modifications, including phosphorylation.

DTEF-1 expression occurs very early in chick embryogenesis (16–18 h), preceding sarcomeric protein expression, and it activates cardiac promoters. As such, DTEF-1 may be an early marker of the myocardial phenotype. The observed spatial pattern of decreasing levels of expression from the cardiac inlet to the ventricular outflow tracts may mark a cardiogenic or differentiation pathway that parallels the direction of flow through the developing chick heart.

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GRANTS

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