Transcription Enhancer Factor 1 Interacts with a Basic Helix-Loop-Helix Zipper Protein, Max, for Positive Regulation of Cardiac α-Myosin Heavy-Chain Gene Expression

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The M-CAT binding factor transcription enhancer factor 1 (TEF-1) has been implicated in the regulation of several cardiac and skeletal muscle genes. Previously, we identified an E-box–M-CAT hybrid (EM) motif that is responsible for the basal and cyclic AMP-inducible expression of the rat cardiac α-myosin heavy chain (α-MHC) gene in cardiac myocytes. In this study, we report that two factors, TEF-1 and a basic helix-loop-helix leucine zipper protein, Max, bind to the α-MHC EM motif. We also found that Max was a part of the cardiac troponin T M-CAT–TEF-1 complex even when the DNA template did not contain an apparent E-box binding site. In the protein–protein interaction assay, a stable association of Max with TEF-1 was observed when glutathione S-transferase (GST)–TEF-1 or GST-Max was used to pull down in vitro-translated Max or TEF-1, respectively. In addition, Max was coimmunoprecipitated with TEF-1, thus documenting an in vivo TEF-1–Max interaction. In the transient transcription assay, overexpression of either Max or TEF-1 resulted in a mild activation of the α-MHC–chloramphenicol acetyltransferase (CAT) reporter gene at lower concentrations and repression of this gene at higher concentrations. However, when Max and TEF-1 expression plasmids were transfected together, the repression mediated by a single expression plasmid was alleviated and a three- to fourfold transactivation of the α-MHC–CAT reporter gene was observed. This effect was abolished once the EM motif in the promoter-reporter construct was mutated, thus suggesting that the synergistic transactivation function of the TEF-1–Max heterotypic complex is mediated through binding of the complex to the EM motif. These results demonstrate a novel association between Max and TEF-1 and indicate a positive cooperation between these two factors in α-MHC gene regulation.

Transcriptional regulation of muscle-specific genes is mediated by a variety of different transcription factors (50). Among them, the skeletal muscle-specific members of the basic helix-loop-helix (bHLH) family of proteins, commonly referred to as the MyoD family (MyoD, Myf-5, myogenin, and MRF-4), are the best understood (49). They play a central role in the induction of the skeletal myogenic program via interaction with other ubiquitously expressed bHLH proteins (E-12/E-47 and Id) and subsequent binding to a well-defined E-box (CANNNTG) consensus sequence (49, 63). Although skeletal and cardiac muscle express many of the same muscle-specific genes, a homolog of the MyoD family of factors has not been identified in cardiac myocytes. However, several lines of indirect evidence suggest that bHLH proteins may participate in cardiac muscle gene expression. Recently, a number of cardiac muscle-specific genes, such as those coding for myosin light chain 2 (MLC-2) (47), cardiac α-actin (46), and α-myosin heavy chain (α-MHC) (30, 44), have been shown to require an intact E-box binding site for optimal activity in a cardiac cell background. Forced expression of either MyoD or Id in cardiac myocytes has indicated that the cardiac cell context is permissive for these bHLH proteins (41, 57). Furthermore, an antibody against the second helix of the myogenic bHLH protein has been found to recognize an epitope in the chicken embryonic heart (38).

Other factors that are described as being necessary for muscle gene regulation include a serum response factor (SRF), which binds to the CC(A/T)_6GG motif (SRE) (26, 42), and the MEF-2 family of factors that bind to the AT-rich sequence found in the enhancer region of many skeletal as well as cardiac muscle genes (25, 67), while some factor(s), such as GATA-4 (31, 45), is required in cardiac but not skeletal muscle cells. Another class of factors necessary for muscle-specific expression of genes in both cardiac and skeletal muscle cell contexts binds to the M-CAT sequence (CATTCCT), originally characterized by Ordahl and associates, for regulation of the cardiac troponin T (cTNT) gene (39). The factor binding to the M-CAT sequence is found to be biochemically and antigenically related to transcription enhancer factor 1 (TEF-1), which was originally found to bind to the GT-IIIc and Sph motifs of the simian virus 40 (SV40) enhancer sequences (22, 64).

TEF-1 is a member of a new family of transcription factors delineated by a homologous DNA-binding domain (DBD), TEA/ATTS, a domain that has been highly conserved throughout evolution (14). A homolog of TEF-1, scalloped, has been identified in Drosophila melanogaster, where it plays an important role in the lineage progression of sensory neuronal development (15). Genes for TEF-1-related factors have been isolated from humans (64), mice (11, 59), rats (24) and chicks (61). In the mouse, ablation of the TEF-1 gene has been shown to result in fetal death due to a defect in cardiac maturation, which is characterized by abnormal thinning of the ventricular wall.
During embryogenesis, TEF-1 appears to be expressed ubiquitously (18), and some evidence implicates it as a possible zygotic factor that is present in embryonic cells at the earliest developmental stages (40). In adult tissue, transcripts of TEF-1 family members are particularly abundant in the kidney, heart, brain, skeletal muscle, and lungs. Furthermore, expression of tissue-restricted TEF-1 isoforms has been documented. The chicken D-TEF-1 isoform has been shown to be expressed at high levels in cardiac muscle compared to other tissues but to be notably absent from skeletal muscle cells (5), and a murine isoform (ETF-1) has been found to be expressed almost exclusively in neuronal tissue (66). Although TEF-1 is considered to be a transcriptional activator, many investigators have observed in in vitro studies that overexpression of recombinant TEF-1 does not activate transcription from the TEF-1 binding site to a level above that generated by endogenous TEF-1 but rather suppresses the endogenous activity at higher concentrations (16, 32, 59). This dominant negative phenotype does not require the sequence-specific binding of TEF-1, since it was also observed for chimeras in which the TEF-1 DBD was deleted or replaced by a heterologous DBD, thus suggesting the possibility of a transcriptional interference squelching effect (16, 32). Furthermore, ectopic expression of recombinant TEF-1 did not activate transcription from a cognate reporter gene in cells that did not contain endogenous TEF-1 (64). This observation led to the proposal that TEF-1 requires cell-specific coactivators, alternatively designated as transcription intermediary factors, that convey cell specificity and a transcriptional activation function to TEF-1. Recently, some negatively acting cofactors of TEF-1 that associate with the TATA-box binding protein (TBP) were partially purified from HeLa and BJA-B lymphoid cells (13, 16, 17). However, the distribution of such factors in other cells capable of directing TEF-1-dependent transcription is not known, and no TEF-1-interacting cellular protein has been identified in muscle cells thus far. Many M-CAT-dependent muscle-specific promoters, such as human β-MHC (23), avian cTNT (37, 39), and mouse ace- tycholine receptor genes (6), have been found to contain a functionally active E-box element in close proximity to the M-CAT binding site. Previously, we have identified an E-box–M-CAT hybrid (EM) motif responsible for the basal and cyclic AMP (cAMP)-inducible expression of the rat cardiac α-MHC gene (30). In this study, we show by using various DNA-protein and protein-protein interaction criteria that two proteins, an M-CAT binding factor, TEF-1, and a bHLH leucine zipper (bHLH-LZ) protein, Max, bind to the EM sequence and that they physically interact with each other both in vitro and in vivo. We also present evidence that the association of Max with TEF-1 does not depend on binding of Max to the E-box binding site. The results obtained from transient transfection analysis demonstrated that the Max–TEF-1 interaction acts as a transcriptional activator to regulate expression of the cardiac α-MHC gene through the EM motif. These are the first results that show a physical interaction of a bHLH-LZ protein (Max) with TEF-1 and that delineate positive cooperation of this interaction in control of gene regulation.

**MATERIALS AND METHODS**

**Construction of plasmids.** The bacterial expression plasmid pGEX-KG was utilized to direct overexpression of full-length Max, mutants of Max, the last 92 amino acids of c-Myc (CTMyc), and TEF-1 proteins. Briefly, the human Max protein was utilized to direct overexpression of full-length Max, mutants of Max, the last 92 amino acids of c-Myc (CTMyc), and TEF-1 proteins. Briefly, the human Max protein was amplified by PCR, digested with Nco I and HindIII, and subcloned into the pGEX-KG vector. The full-length TEF-1 protein was amplified from pBS-TEF-1 (59) by PCR, digested with Xhol and XbaI, and subcloned into the XbaI and Xhol sites of the pGEX-KG vector. The glutathione S-transferase (GST)–Max, GST-CTMyc, and GST–TEF-1 expression plasmids were subsequently transformed into HB101 cells for inducible production of protein. The mammalian expression plasmid p53-V-TEF-1 has been described elsewhere (1). The TEF-1 expression plasmid, kindly furnished by Paul Simpson, contains rat TEF-1 cDNA under the control of the cytomegalovirus promoter (parental vector, pXJ40). The plasmid pMP.EMmt-1CAT and pMP.EMmt-2.CAT contain point mutations in the E-box and M-CAT elements of the EM motif, respectively (see Fig. 12). The construction of these plasmids has been described elsewhere (30).

**Preparation of nuclear extracts and performance of EMSAs.** Nuclear extracts were prepared from rat hearts and skeletal muscle by the method of Dignam et al. (21), with slight modifications as described previously (30). For the electrophoretic mobility shift assay (EMSA), double-stranded oligonucleotides were 5′-end labeled with T4 polynucleotide kinase (Gibco BRL) and [γ-32P]ATP (56). The analytical binding reaction mixture was incubated at room temperature for 15 to 20 min prior to addition of the labeled probe. The Max–TEF-1 study was raised at The University of Chicago against a synthetic peptide corresponding to the last 21 amino acids (from amino acid 140 to amino acid 160) of the human Max protein. The anti-TEF-1 antibody (P-1 antibody) was raised against the first 24 amino acids of the human TEF-1 protein (a gift from C. Chambon).

**Immunoblot analysis.** EMSAs were performed with the same gel used for the labeled and unlabeled oligonucleotide probes. The DNA-protein complex that occurred with the labeled probe was located by autoradiography of the wet gel, and the gel piece corresponding to the protein complex with unlabeled probe was excised and submerged in Lactose protein sample buffer supplied with the gel. The gel was electroeluted in 0.1% Triton X-100. The integrity of the GST fusion proteins bound to the glutathione-agarose beads were washed thoroughly with PBS containing 0.1% Triton X-100, and the bacterial lysate was analyzed by resolution of proteins by SDS-polyacrylamide gel transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp.), probed with the appropriate antibodies, and developed according to ECL Western blotting protocols (Amersham Corp.).

**Overexpression and purification of GST fusion proteins.** The GST fusion proteins were expressed in bacteria and purified as described previously (3, 29). In brief, bacteria harboring plasmid pGSTM-MAX, pGST-TEF-1, or pGST-CTMyc were grown overnight in Luria-Bertani medium supplemented with ampicillin. The next morning, cells were diluted 1:10 with fresh medium, grown to an optical density at 600 nm of 0.5 to 0.75, and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to direct expression of fusion proteins. After 3 to 7 h of expression the GST fusion proteins, cells were harvested by centrifugation at 4°C, washed with PBS containing 0.1% Triton X-100 and resuspended in phosphate-buffered saline (PBS) containing protease inhibitors, and sonicated for a total elapsed time of 120 s. The bacterial lysate was solubilized by the addition of Triton X-100 to a final concentration of 1% and centrifuged at 13,000 × g for 4°C to remove insoluble material. Glutathione-agarose beads were added to the soluble supernatant fraction, and the binding of GST fusion proteins was allowed to occur at 4°C for 30 min. The beads were pelleted in an Eppendorf centrifuge at 4,000 × g for 2 min, and the GST fusion proteins bound to the glutathione-agarose beads were washed thoroughly with PBS containing 0.1% Triton X-100. The integrity of the GST fusion proteins bound to the beads was analyzed by resolution of proteins by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining; known amounts of bovine serum albumin were included on the same gel for determination of the yield of full-length fusion proteins.

**Affinity purification of Max with GST fusion proteins.** We sought to use the selective interaction of the last 92 amino acids of the c-Myc protein with Max (7) as a means of depleting or reducing the presence of Max in nuclear extracts of the rodent rat heart. Five milligrams of GST, GST-CTMyc, and GST–TEF-1 bound to glutathione-agarose beads was incubated with 40 μg of the neonatal rat heart nuclear extract in 1 × DNA binding buffer for 3 h at 4°C with continuous rocking. Glutathione-agarose beads lacking the GST fusion protein were also incubated with the nuclear extract to serve as a negative control. After 3 h of incubation, beads were pelleted at 14,000 × g for 2 min and the supernatant was collected and used directly in the EMSA. An identical procedure was used for depletion of Max from nuclear extract, with GST–TEF-1 being employed as the interacting protein. To detect the interaction of Max with the GST fusion protein, the pelleted beads were washed five times with 1 ml of 1 × DNA binding buffer,
suspended in 2× Laemlli buffer, and subjected to subsequent Western blot analysis.

**In vitro characterization of binding of Max to TEF-1.** The TNT-coupled rabbit reticulocyte lysate system (Promega) was used to translate the pBS-Max and pBS-TEF-1 plasmids. After translation, the specific incorporation of [35S]methionine into proteins was determined by trichloroacetic acid precipitation and the integrity of the translated proteins was checked by SDS-PAGE and autoradiography. For the in vitro binding assay, [35S]-labeled proteins were incubated with 2 to 3 μg of GST or GST fusion proteins (GST–TEF-1 or GST-Max) on glutathione-agarose beads in 1× protein interaction buffer (PIB; 20 mM HEPES [pH 7.5], 75 mM KCl, 1 mM EDTA, 2 mM MgCl₂, 2 mM DTT, and 0.5% Nonidet P-40 [NP-40]) for 2 h at 4°C with continuous rocking. The beads were pelleted and washed five times with 1× PIB. The bound proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE.

**Far-Western analysis.** The GST and GST–TEF-1 proteins were overexpressed in bacteria (Escherichia coli DH5α) and isolated according to standard protocols as described before (29). The TEF-1 protein was cleaved from the fusion protein by thrombin digestion, and the full-length TEF-1 protein was separated from smaller degradation products by filtration through the Centricon 30 size exclusion spin column (Amicon). The integrity of proteins to be blotted on the nitrocellulose (NC) membrane was checked by SDS-PAGE and Coomassie blue staining. For the far-Western experiment, a bacterial expression plasmid, pGEX-KG to create the pGEX-cAMP plasmid. Subsequently, an NcoI-SacI fragment (containing full-length Max cDNA) was isolated from pBS-Max (1) and subcloned into the NcoI and SacI sites of the plasmid GEX-cAMP to direct expression of GST-cAMP-Max. The GST-cAMP-Max fusion protein was overexpressed in DHLα cells, and then it was isolated and labeled with the catalytic subunit of the bovine cAMP-dependent PK-A (Sigma) essentially as described before (35). The labeled GST-cAMP-Max fusion protein was eluted from the beads, and 2 × 10⁵ cpm of protein probe per ml was added to the NC blot that contained 50 ng of each protein (TEF-1, GST–TEF-1, His-Max, and GST) in 1× hybridization buffer (20 mM HEPES [pH 7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, and 0.05% NP-40). The NC blot was incubated with the 32P-labeled GST-cAMP-Max fusion protein overnight at 4°C. The unbound labeled protein was removed by four washes with 1× hybridization buffer at room temperature. The NC blot was wrapped in Saran Wrap and subjected to autoradiography at –70°C for 4 to 12 h.

**Cell culture and transfection.** Primary myocytes were cultured from 18-day-old fetal rat hearts (see reference 30). After differential plating to eliminate nonmuscle cells, myocytes were plated at a density of 2 × 10⁵ cells/100-mm-diameter culture dish (Falcon brand; Becton Dickinson Labware) precoated with 0.1% gelatin in Ham’s F-12 medium (Gibco BRL) with 5% calf serum. Cultures generally consisted of more than 90% myocytes, as measured by immunocytofluorescence with antibinomyosin antibody (39). More than 90% of the cells began to contract spontaneously within 24 h after plating. Ltk⁻ fibroblasts and Cos7 cells were grown in growth medium containing Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂. All culture media contained penicillin (5 mg/ml), streptomycin (5 mg/ml), and neomycin (100 mg/ml).

After 48 h in culture, primary cultures of cardiac myocytes were transfected with 10 μg of DNA/plaque by use of a lipofectamine reagent (Gibco BRL). For cotransfection experiments, 5 μg of the α-MHC–CAT reporter plasmid was transfected with pSV-Max, pCMV-TEF-1, or a parental expression vector lacking cDNA. All transfections reaction mixtures contained 1 μg of the pCMV-βGal reference plasmid. The Ltk⁻ fibroblasts were transfected at 40% confluence by a similar procedure. After 48 h of transfection, cells were harvested, cell lysates were prepared, and CAT and β-galactosidase assays were performed on the same cell extract samples (54).

**Coimmunoprecipitation of Max with TEF-1.** A Flag-tagged TEF-1 expression plasmid was constructed by subcloning a PCR fragment of TEF-1 from pBS-TEF-1. The 5’ primer (5’-GG CCT GGT ACC ATG GAC TAC AAG GAC-3’) and the 3’ primer (5’-GGG CTC GAG TCA GTC CTT CAC AAG CTT GTA GAT-3’) were used to amplify on a 5% polyacrylamide gel. The PCR product was cloned into pKpl and XhoI sites of the pCMV-5 vector. Cos7 cells, at a density of 10⁵ cells/ml, were transfected with the expression plasmids pCMV-Flag-TEF-1 and pSV-Max by the lipofectamine procedure. Following 36 h of transfection, cells were washed with ice-cold PBS and harvested in a high-salt (100 mM NaCl) lysis buffer. The cells were allowed to lyse on ice for 10 min after collection and were further disrupted by forcing them through a 22-gauge needle several times. The lysed-cell extract was spun to remove cell debris, and the supernatant (whole-cell extract) was transferred to a fresh tube. It was checked by Western blot analysis for the expression of ectopically expressed proteins. For immunoprecipitation of proteins, the whole-cell extract was incubated with 2 μl of MZ (Flag) antibody.
RESULTS

Binding of TEF-1 and Max proteins to the α-MHC EM hybrid motif. Previous studies have led to the identification of a 13-bp EM hybrid motif that is responsible for the basal as well as the cAMP-inducible expression of the α-MHC gene (30). To determine cardiac muscle factors which bind to this motif, an EMSA was carried out with neonatal rat heart nuclear extract (N.E.). As shown in Fig. 1, two specific DNA-protein complexes of different mobilities and intensities were detected, a slowly migrating major complex, C₁, and a faster-migrating minor complex, C₂. The C₁ complex has a gel mobility identical to that of the complex that occurs with the cTNT M-CAT oligonucleotide (Fig. 1B). In the competition assay, an excess of oligonucleotides corresponding to the cTNT M-CAT and SV40 GT-IIc motifs, which are known to bind TEF-1, competed successfully for both EM-protein complexes (lanes 4 and 12 and 13), suggesting involvement of a TEF-1-related protein in both of these complexes. In order to test whether an E-box binding protein could also be a part of EM complexes, we synthesized two different EM mutant oligonucleotides, EM-mt-1 and EM-mt-2, containing mutations in the E-box and M-CAT elements, respectively (Fig. 1A), and used them either as competitors or as labeled probes in EMSAs. As shown in Fig. 1B, the EM-mt-2 oligonucleotide retained the ability to compete for the factors binding to the α-MHC EM motif (lanes 10 and 11) but the EM-mt-1 oligonucleotide did not (lanes 8 and 9), thus documenting the importance of the E-box element of the EM motif for complex formation. This observation was further supported by the fact that the complex formed by the labeled EM-mt-2 oligonucleotide was inhibited by an excess of the unlabeled E-2 (Fig. 3A, lanes 9 and 10) as well as EM (Fig. 3A, lanes 5 and 6) oligonucleotides but not by the M-CAT oligonucleotide (Fig. 3A, lanes 7 and 8). However, when the EM-mt-1 oligonucleotide was used as a labeled probe, no distinct DNA-protein complex formation occurred (Fig. 3B). This is consistent with our previous results of an in vitro footprint analysis indicating that methylation of G residues of the E-box element strongly interferes with a factor(s) binding to the EM motif (30). Together, these results indicate that both the M-CAT and the overlapping E-box sequences are required for the TEF-1-related protein to bind to the EM motif. On the other hand, interaction of the E-box binding protein is independent of the presence of the perfect M-CAT site of this motif. These results are also in agreement with a recently published report documenting the importance of the flanking bases, including the E box, of the M-CAT core for the formation of a TEF-1-related complex on the M-CAT site of a muscle gene promoter (37).
to the binding reaction greatly inhibited the formation of C1-EM and C2-EM complexes, and a small portion of the C2-EM complex was also found to be supershifted (arrow, lane 3), suggesting the presence of a TEF-1-related protein in both complexes. The cTNT M-CAT protein complex was also abolished and partly supershifted by the TEF-1 antibody (Fig. 4A, lane 8). We also tested the effect of this TEF-1 antibody on cardiac nuclear extract with increasing amounts of anti-Max antibody and 2,000-fold-diluted horseradish peroxidase-labeled anti-Max antibody (TEF-1 Ab) (panel A, lanes 3 and 8), anti-Max antibody (Max Ab) (panel B, lanes 3 and 5), or preimmune serum (panel A, lanes 4 and 9; panel B, lane 4). For panel A, labeled probes were also incubated with antibodies in the absence of nuclear extract (lanes 5 and 10). The arrows represent supershifted C1 complex in lane 3 and the M-CAT complex in lane 8 with the TEF-1 antibody. Competitors are as given in the legend to Fig. 1. MBS denotes an oligonucleotide (shown in Fig. 5) containing a high-affinity Myc/Max protein binding site.

To characterize proteins binding to the α-MHC EM motif, we carried out EMSAs in which the cardiac nuclear extract was preincubated with protein-specific antibodies. In these experiments, DNA-protein complexes formed with cTNT M-CAT and cardiac α-actin E-2 oligonucleotides were used as positive controls for TEF-1 and E-box binding proteins, respectively. As shown in Fig. 4A, addition of anti-human TEF-1 antibody to the binding reaction greatly inhibited the formation of C1-EM and C2-EM complexes, and a small portion of the C2-EM complex was also found to be supershifted (arrow, lane 3), suggesting the presence of a TEF-1-related protein in both complexes. The cTNT M-CAT protein complex was also abolished and partly supershifted by the TEF-1 antibody (Fig. 4A, lane 8). We also tested the effect of this TEF-1 antibody on cardiac nuclear extract with increasing amounts of anti-Max antibody and 2,000-fold-diluted horseradish peroxidase-labeled anti-Max antibody (TEF-1 Ab) (panel A, lanes 3 and 8), anti-Max antibody (Max Ab) (panel B, lanes 3 and 5), or preimmune serum (panel A, lanes 4 and 9; panel B, lane 4). For panel A, labeled probes were also incubated with antibodies in the absence of nuclear extract (lanes 5 and 10). The arrows represent supershifted C1 complex in lane 3 and the M-CAT complex in lane 8 with the TEF-1 antibody. Competitors are as given in the legend to Fig. 1. MBS denotes an oligonucleotide (shown in Fig. 5) containing a high-affinity Myc/Max protein binding site.

Next, to identify the E-box binding protein that participates in these complexes, we performed a similar experiment using anti-Max antibody. As shown in Fig. 4B, incubation of the nuclear extract with this antibody also inhibited formation of both EM probe C1 and C2 complexes; however, no change was seen with the preimmune serum. As a positive control, the effect of Max antibody on cardiac α-actin E-2 protein complex formation was also tested, and as indicated in Fig. 4B, the formation of this complex was inhibited as well. These results suggest that in addition to TEF-1, a Max-related protein is also a part of both the C1 and C2 complexes of the α-MHC EM motif. Antibodies against c-Myc and myogenin were tested as

FIG. 4. TEF-1 and Max proteins are part of both the C1 and C2 complexes of the α-MHC EM probe. The cTNT M-CAT and cardiac α-actin E-2 templates were utilized as positive controls for binding of TEF-1 and Max proteins, respectively. In the EMSA reaction, cardiac nuclear extract (N.E.) was preincubated with 2 μl of anti-TEF-1 antibody (TEF-1 Ab) (panel A, lanes 3 and 8), anti-Max antibody (Max Ab) (panel B, lanes 3 and 5), or preimmune serum (panel A, lanes 4 and 9; panel B, lane 4). For panel A, labeled probes were also incubated with antibodies in the absence of nuclear extract (lanes 5 and 10). The arrows represent supershifted C1 complex in lane 3 and the M-CAT complex in lane 8 with the TEF-1 antibody. Competitors are as given in the legend to Fig. 1. MBS denotes an oligonucleotide (shown in Fig. 5) containing a high-affinity Myc/Max protein binding site.

To verify this observation further, several other experiments were carried out with either competitor oligonucleotides containing an E-box binding site or anti-Max antibody. As shown in Fig. 5B, an oligonucleotide containing a previously characterized high-affinity MBS competed effectively for the M-CAT–protein complex (lanes 3 and 4), but the mutant oligonucleotide did not (lane 8). It should be noted that the cardiac α-actin E-2 oligonucleotide also competed for the M-CAT–protein complex (Fig. 2, lane 5), thus suggesting that an E-box binding protein is part of this complex. This observation was also supported by the fact that in the EMSA, preincubation of cardiac nuclear extract with increasing amounts of anti-Max antibody inhibited M-CAT complex formation in a con-

FIG. 5. Max is a part of the cardiac α-actin E-2, α-MHC EM, and cTNT M-CAT–protein complexes. (A) Western blot analysis. DNA-protein complexes obtained with unlabeled probes by EMSA were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (as described in Materials and Methods). Western blot analysis was performed with 1,000-fold-diluted anti-Max antibody and 2,000-fold-diluted horseradish peroxidase-labeled anti-rabbit antiserum. The reaction carried out with the EM-mt-1 oligonucleotide (which does not form a complex) was used as a negative control (Mock). Fifty nanograms of His-Max was used as a positive control. With the EM probe, results show the presence of Max in the C2 complex; however, Max was also detected in the C1 complex (data not shown). (B) EMSA. The end-labeled cTNT M-CAT probe was incubated with 4 μg of cardiac nuclear extract, and complex formation was analyzed on a 5% polyacrylamide gel. (C) Sense strand of competitor oligonucleotides containing an MBS and an MBS with a mutation (MBSmt).
centration-dependent manner (Fig. 6A). Similar results were obtained when Max was immunodepleted from the nuclear extract (Fig. 6B, lane 2). Furthermore, addition of His-Max to Max-depleted nuclear extract strengthened the M-CAT–protein complex (Fig. 6B, lane 3) while Max by itself did not bind to the M-CAT oligonucleotide (Fig. 6B, lanes 4 and 5). These results strongly suggest that in addition to TEF-1, Max (or a Max-related protein that is indistinguishable from Max in terms of immunoreactivity and molecular mass) is a part of the cTNT M-CAT–protein complex, and they provide indirect evidence for an interaction between these two proteins. From these results, we also conclude that the cTNT M-CAT complex is identical to the C1 complex of the α-MHC EM motif; both contain TEF-1 as well as Max proteins. The C2 complex of the EM motif is a multiprotein complex that includes TEF-1, Max, and another, unidentified factor(s) that may associate with them.

Among the bHLH-LZ proteins, the Myc protein has been shown to heterodimerize specifically with Max, a process involving the HLH-LZ domains of both proteins (36). Therefore, in order to obtain another line of evidence, we sought to deplete Max from the nuclear extract by taking advantage of its ability to interact with Myc. In this experiment, nuclear extract was incubated with the GST–CTMyc fusion protein, which contained the last 92 amino acids (comprising the entire bHLH-LZ region) of the Myc protein, and then removal of the GST fusion protein by use of glutathione-agarose beads, the supernatant was analyzed by EMSA. Nuclear extract treated with GST or glutathione-agarose beads alone was used as a negative control. As shown in Fig. 7A, the intensity of the DNA-protein complex formed with either M-CAT, EM, or E-2 probes was diminished markedly in the GST-CTMyc-treated nuclear extract (lanes 2, 7, and 12). However, no change was observed with either GST or agarose beads alone, suggesting that the protein physically interacting with the last 92 amino acids of the Myc protein must be part of these complexes.

Because our previous experiments had raised the possibility of Max binding to TEF-1, we performed an identical experiment using GST–TEF-1 to deplete the nuclear extract of Max. As shown in Fig. 7A, incubation of the nuclear extract with GST–TEF-1 and subsequent removal of the GST fusion protein-agarose beads almost completely abolished the formation of complexes with all three different probes used in this study (lanes 4, 9, and 14). As before, no change from the GST or glutathione-agarose-treated nuclear extract was observed, thus suggesting that proteins required for formation of complexes with these probes must be removed because of their physical interaction with TEF-1. To find out whether Max was indeed removed from the nuclear extract, we confirmed by Western blot analysis the presence of Max on glutathione-agarose beads on which GST-CTMyc or GST–TEF-1 was immobilized (Fig. 7B). The results suggest a direct interaction of Max with TEF-1.

TEF-1 interacts with Max. To test the binding specificity of TEF-1 for Max directly, we examined the ability of in vitro-translated proteins to bind to bacterially expressed GST–TEF-1 or GST-Max fusion proteins. In vitro translation reactions programmed with plasmids encoding either the full-length Max or TEF-1 were carried out. As a negative control, a parallel reaction was performed in which rabbit reticulocyte lysate was programmed under identical reaction conditions but
without any plasmid. After translation, glutathione-agarose beads that were bound with GST or GST fusion proteins were incubated with the translated proteins. The beads were washed extensively, and bound proteins were analyzed by SDS-PAGE (Fig. 8 and 9). The [35S]methionine-labeled Max was retained on the beads that contained GST–TEF-1 (Fig. 8, lane 3). Reciprocally, [35S]methionine-labeled TEF-1 was retained on the beads that contained GST-Max (Fig. 9C, lane 9), whereas neither protein interacted with the GST protein alone. This indicated that Max and TEF-1 proteins were retained on the matrix through the TEF-1 and Max segments of the GST fusion proteins, respectively. To examine which regions of Max were required for interaction with TEF-1, we tested two different Max mutants, one with an 18-amino-acid amino-terminal deletion and the other with a 48-amino-acid carboxy-terminal deletion, for their ability to interact with TEF-1. As shown in Fig. 9C, both GST-ΔN-Max (lane 10) and GST-ΔC-Max (lane 11) also successfully retained [35S]methionine (lanes 7 to 11). The translated proteins were incubated with GST or different GST-Max fusion peptides on beads as indicated above each lane. Proteins bound to GST or GST fusion peptides were analyzed on an SDS-polyacrylamide gel. R.R. programmed without any plasmid and R.R. with pBS-Max (R.R.-Max) were used as negative and positive controls, respectively.

We used another protein-protein interaction assay (far-Western analysis) to determine the in vitro Max and TEF-1 interaction. In this experiment, proteins were resolved by SDS-PAGE, transferred to NC membranes, and then hybridized with 32P-labeled GST-cAMP-Max fusion protein. As before, interaction of Max with itself and no interaction with GST were used as positive and negative controls, respectively (Fig. 10). In this assay, also, we found that Max strongly interacts...
Identification of binding of Max and TEF-1 to the α-MHC EM motif and their physical interaction raises the possibility of their cooperation in gene regulation. To determine a functional role for the TEF-1 and Max proteins, expression plasmids that encode full-length rat TEF-1 or human Max protein were cotransfected in primary cultures of cardiac myocytes with a wild-type or EM element-mutated promoter-reporter construct containing a fragment consisting of bp −156 to bp +30 of the α-MHC gene. Overexpression of either TEF-1 or Max resulted in a concentration-dependent effect on the expression of the reporter plasmid (pMP.EM.CAT). A modest activation at low concentrations was followed by repression at higher concentrations of the expression plasmids (Fig. 12B). We also tested whether titration of a fixed amount of either expression plasmid with increasing concentrations of the promoter-reporter construct could potentiate CAT expression. However, no further change in CAT expression by either the Max or the TEF-1 protein was observed with increasing concentrations of the reporter plasmid, indicating that the concentration-dependent biphasic effect of Max and TEF-1 is not due to the ratio of the expression plasmid to the promoter-reporter construct. Rather, it seems that some other endogenous limiting factor(s) present in myocytes controls the trans-regulatory effect of these two proteins.

To test whether Max and TEF-1 together could cooperate positively in gene regulation, both Max and TEF-1 expression plasmids were transfected together with the wild-type (pMP.EM.CAT) reporter plasmid in cardiac myocytes. Figure 12B shows that when a fixed amount of the Max expression plasmid was transfected together with different concentrations of the TEF-1 plasmid, a three- to fourfold transactivation of the reporter plasmid was observed. Similar results were obtained in a reverse experiment in which a fixed amount of the TEF-1 expression plasmid was cotransfected with increasing concentrations of the Max plasmid. A maximal transactivation effect of the two expression plasmids was observed when they were used in equal amounts; changing the concentration of either expression plasmid from this level resulted in a lesser transactivation effect. These data suggest that a particular stoichiometric level of TEF-1 and Max is important for an optimal transactivation effect. Furthermore, no effect of either expression plasmid was observed when either E-box or M-CAT site-mutated promoter-reporter constructs were used, indicating that a cooperative effect of these two proteins must occur through their binding to the EM motif. We also tested whether this transactivation effect of Max and TEF-1 was restricted to cardiac myocytes. A similar experiment was performed with Ltk<sup>−</sup> fibroblasts, and the results indicated that this cell background is also permissive for the cooperative effect of Max and TEF-1 proteins (data not shown). Thus, together these results strongly suggest that Max and TEF-1 can associate in vivo and function as transcriptional activators in different cell backgrounds.
DISCUSSION

Our objective in this study was to identify factors binding to the previously characterized E-box–M-CAT hybrid motif that are required for the optimal expression of the α-MHC gene in cardiac myocytes. This motif has also been shown previously to be responsible for the cAMP-induced (30) and contractile-activity-mediated (48) up-regulation of α-MHC gene expression. Several lines of evidence presented here indicate that at least two factors present in neonatal rat cardiomyocytes that interact with the α-MHC EM sequence are related to the TEF-1 and Max proteins. Our data also demonstrate that Max is a part of the complex generated by the avian cardiac α-actin E-2 and cTNT M-CAT sequences, which were previously shown to be necessary for muscle-specific gene regulation (39, 46). More importantly, in this study we have established that TEF-1 and Max physically interact with each other and that this heterotypic interaction is capable of transactivating the expression of the α-MHC gene in vivo through the E-box–M-CAT hybrid motif.

TEF-1 is considered to be a transcription factor that is primarily responsible for transcriptional activation by binding to the CATTCCT sequence found in certain muscle-specific and viral genes. The TEF-1 binding element in many genes besides the α-MHC gene has been found to be located either overlapping or in close proximity to an E-box binding site (Table 1). In the rat acetylcholine receptor β-subunit (6), avian cTNT (39), and human β-MHC (23) genes, an adjacent E-box element has been shown to cooperate in a synergistic fashion with the M-CAT element to control muscle-specific gene expression. Similarly, the TEF-1 binding site GT-Iic in the SV40 early promoter region, which exhibits cell-specific activity of the viral gene, has been shown to be partially occupied by proteins binding to the adjacent GT-Iib sites (65). This GT-Iib complex could be successfully abolished by the μE3 motif of the immunoglobulin H enhancer, which contains a perfect E-box binding element (65). These studies have provided indirect evidence of a mutual relationship between the E-box binding protein and TEF-1 for gene regulation. The results presented in this study are consistent with these previous observations and indicate that in some (if not all) cases, TEF-1-dependent gene regulation could be controlled by an adjacent E-box binding protein.

The role of the E box and its binding factors, which may confer cardiac-specific gene expression, is based on the paradigm of skeletal muscle genes that are regulated by cell-specific bHLH proteins. Skeletal muscle-specific bHLH proteins are not detected in cardiac myocytes; however, several earlier reports have suggested the participation of some other E-box binding factor belonging to the bHLH or bHLH-LZ family of proteins in cardiac muscle gene regulation (30, 38, 41, 44, 46, 47, 57). Our work presented in this report formally demonstrates that a bHLH-LZ protein, Max, plays a role in the regulation of the cardiac α-MHC gene and also implicates its involvement in cardiac α-actin and cTNT gene expression.

Max is a nuclear phosphoprotein that was originally cloned...
as a specific heterodimeric partner of Myc (10). However, unlike Myc, which is highly regulated during the cell cycle, Max is a relatively stable protein (half-life, ≥15 h) that is expressed in proliferating as well as in differentiated cells, including cardiac and skeletal muscle cells (8, 62, 68). Max has been shown to bind to the E-box element either as a homodimer or as a heterodimer by interacting with other members of the bHLH-LZ family of proteins, namely, the Myc family of proteins (7) or two other recently identified proteins, Mad and Mxi (4, 68). Mutational analysis of Max has revealed that the HLH-LZ region of this protein interacts with similar domains of other partners to form a stable heterodimeric complex (36). However, based on the amino acid sequence homology of the TEF-1 and bHLH-LZ family of proteins, TEF-1 does not contain a putative HLH-LZ domain that could mediate its interaction with Max. Nevertheless, several lines of evidence presented in this study strongly demonstrate an interaction between Max and TEF-1 that occurs in vitro as well as in vivo.

Presence of Max in the cTNT M-CAT complex: indirect evidence of TEF-1–Max interaction. The cTNT M-CAT oligonucleotide used in this study contains a perfect TEF-1 binding site; however, it apparently lacks an E-box binding site. Yet the formation of a complex by this oligonucleotide was inhibited by an excess of cold E-2 as well as MBS competitor oligonucleotides containing a high-affinity E-box binding site, even though these oligonucleotides do not contain a TEF-1 binding site. This raised the question of whether an E-box binding protein could also be present in the M-CAT complex. Data obtained from experiments involving, for instance, reduction or abolition of the complex due to depletion of Max from the nuclear extract by either GST-CTMyc or immunoprecipitation by Max antibodies, as well as detection of Max at the M-CAT site by Western blot analysis, confirmed this possibility and revealed that the Max protein is indeed present in the M-CAT protein complex. Max by itself does not bind to the M-CAT oligonucleotide, thus suggesting that the binding of TEF-1 to the M-CAT site might recruit Max for formation of a TEF-1–Max heterotetramic complex. An analogous situation has been described for the interaction of other heterologous transcription factors, such as SAP-1 or Phox-1 with the SRF-SRE complex for the c-fos promoter (20, 28) and association of MEF-2 with the bHLH myogenic factors–E-box DNA complex for skeletal muscle gene promoters (43). This type of recruitment could be a general mechanism for promoter communication by which heterologous transcription factors are brought into contact for mutual cooperation.

TEF-1 and Max interaction: a novel association for the bHLH-LZ protein. In some cases, the interaction of two heterologous factors has been shown to be dependent on the sequence-specific binding of one factor to DNA, as is the case for binding of SAP-1 to the SRF-SRE complex (20). This condition does not seem to apply to the interaction of Max with TEF-1, because in both the in vitro and the in vivo protein–protein interaction assays, a stable association of Max with TEF-1 was observed. Thus, these findings suggest that Max–TEF-1 interaction can take place in the solution when binding of either factor to a cognate DNA binding site is not possible. Until now, no transcription factor lacking the HLH-LZ motif, other than TEF-1 (this work), has been found to interact with Max. Attempts to delineate the region of the Max protein that interacts with TEF-1 have shown that wild-type Max, ΔN-Max mutants, and ΔC-Max mutants all retain the ability to interact

| Gene | Sequence | Reference |
|------|----------|-----------|
| a-MHC (r) | -56 C.AGA.CAC.GTT.GAA.TGT -41 G.TCC.GTC.CAC.CCT.ACA | 30 |
| cTNT (c) | -106 CA.CAA.GTG.TTG.CAT.CGC.TCT -87 GT.GTY.CAC.AAC.GTA.AGA.AGA | 39 |
| β-MHC (h) | -302 TCC.TGC.CAG.CTG.TGG.AAT.GTG.AG -280 AGG.ACG.GTC.GAC.AGC.TTA.CAC.TC | 23 |
| Cardiac actin (m) | -394 G.CAT.GTG.ACT.CAT.TGT.CC -377 C.GTAC.TGA.GTA.ACA.GG | 42 |
| Cardiac MLC-2 (c) | -44 CAT.GGG.GTT.ATT.TTT.AGC.CTG.GAA.TG -19 GYT.CACA.CTC.GTG.GAA.GGA.GCA.TAC | 55 |
| Embryonic MHC (r) | -346 CAA.GTG.GAG.CAG.CCT.CTT.CTG.ATG -321 GTG.CAC.CTC.GTG.GAA.GGA.GCA.TAC | 12 |
| AchR, β-subunit (r) | -59 CAA.CAG.GTG.CAC.ATT.CCT.G -41 GYT.GTC.CAC.GTG.TAA.GGA.C | 6 |
| AchR, β-subunit (m) | -55 CAA.CAG.GTG.CAC.ATT.CCT.GGG -35 GTT.GTC.CAC.GTG.TAA.GGA.CCC | 53 |
| AchR, γ-subunit (c) | -67 CAT.TGC.TGC.TGA.CAA.CAG.ATG -44 GAT.ACG.ACG.ACT.CCT.GTC.GAC.TAC | 33 |
| AchR, α-subunit (c) | -110 CCT.CAC.CTG.TCA.TGC.CTG.GAA -90 GGA.GTC.GAC.AGT.ACG.GAC.OTT | 52 |
| ANF (r) | -90 CG.CAA.GTG.ACA.GAA.TGG.GGG.G -69 GC.GTAC.CAT.GTG.CCT.ACC.CCT.C | 58 |
| ANF (r) | -1144 G.ATA.CAT.GTG.GAT.GTG.AA -1127 CG.TAT.GTAC.TGA.CAC.TC | 58 |
| MCK (m) | -440 TCC.TCC.CAG.CTG.CTA.ATG.CTC -420 AG.AG.GTG.GAC.GAT.TAC.GAG | 60 |
| SV40* | 280 TC.GGG.CAG.CTG.TGG.AAT.GTC.TGT 257 AG.CCC.GTC.GAC.AGG.TTA.CAC.ACA | 65 |

* The m, r, c, and h designations refer to mouse, rat, chicken, and human, respectively. The asterisks denote genes for which the functional significance of both the E-box and M-CAT sequences has been characterized. Abbreviations: AchR, acetyl choline receptor; ANF, atrial natriuretic factor; MCK, muscle creatine kinase.

The E-box elements of the different genes are aligned for comparison and are shown in boldface letters, whereas M-CAT elements are underlined.
with the TEF-1 protein. These results led us to propose that the region of Max that binds to TEF-1 must be in the bHLH-LZ region of the protein; however, this remains to be demonstrated by using specific mutations in this region of the protein.

Data obtained by far-Western analysis, in which radiolabeled GST-cAMP-Max was found to interact with TEF-1 and GST–TEF-1 fusion proteins even when these proteins were immobilized on the NC blot, indicate that this interaction is stable and that the secondary structures of TEF-1 preserved in this assay are sufficient for Max and TEF-1 interaction. Moreover, these results provide insight into the putative region of TEF-1 that may mediate dimerization with Max. The GST–TEF-1 fusion protein that was eluted from glutathione-agarose beads and resolved by SDS-PAGE produced two major bands (44 and 70 kDa) when duplicate blots were probed with GST and TEF-1 antibodies (data not shown), indicating that both the full-length and the truncated fusion proteins on the blot contained the GST moiety. In the far-Western experiment, we observed that the radiolabeled GST-cAMP-Max fusion protein interacts not only with the full-length GST–TEF-1 but also with the 44-kDa truncated protein that includes the GST moiety; however, no interaction was seen with the GST protein alone. If the 26-kDa GST moiety is subtracted from the 44-kDa truncated GST–TEF-1, then the TEF-1 protein contributes about 18 kDa, which can be deduced to be roughly 180 amino acids of the TEF-1. Although mapping of the specific region of TEF-1 requires the use of deletion mutants of the protein, we believe, based on our far-Western data, that the first 180 amino acids of TEF-1 may be sufficient to mediate the newly discovered interaction between the TEF-1 and Max proteins.

Positive cooperation between Max and TEF-1 for gene regulation. The results obtained from our transfection analysis have demonstrated that the α-MHC promoter activity is a function of the relative amounts of the Max and TEF-1 proteins. Among the different Max complexes known thus far, only the Max-Myc heterodimer has been shown to activate transcription, whereas others, like the Max-Max, Max-Mad, and Max-Mxi complexes, have been documented to repress transcription from the E-box binding site (4). In this study, two different lines of evidence argue against participation of Myc in the Max-induced transactivation of the α-MHC–CAT plasmid. First, when anti-c-Myc antibody was used in the gel shift assay, we did not find evidence of involvement of Myc in EM-protein complex formation. Second, an identical DNA-protein complex was observed with nuclear extracts of tissues, such as adult rat heart and So18 myotubes, in which Myc expression was positive, and yet the EM motif was characterized as a positive cis regulatory element (30). Importantly, in this study we have found that the repression of the α-MHC promoter mediated by either Max or TEF-1 is alleviated once both expression plasmids are transfected together. Furthermore, a linear increase in transactivation was observed when the concentration of one expression plasmid was kept constant while the concentration of the other plasmid was gradually increased. These results provide strong support for the concept that the Max and TEF-1 interaction observed in this study, based on various criteria, represents a physiologically relevant association.

Recently, interaction of TEF-1 with the large T antigen has been shown to result in the transcriptional activation of the SV40 late promoter (9). However, this transactivation has been suggested to be a function of the derepression of the promoter activity, due to inhibition of DNA binding by TEF-1 resulting from TEF-1–T-antigen interaction. A similar mechanism does not seem to be applicable for transactivation of the α-MHC gene by TEF-1–Max interaction, because TEF-1 binding sites of the viral late promoter and muscle gene elicit different characteristics upon mutation: activation of the former, but inhibition of the latter. Furthermore, in contrast to the TEF-1–T-antigen interaction results, our results indicate that Max–TEF-1 interaction strengthens the binding of TEF-1 to the M-CAT site.

How does the interaction between Max and TEF-1 result in a synergistic activation of the α-MHC gene promoter? Max is a short protein of only 160 amino acids and does not contain a transactivation domain (7). Therefore, any transactivation activity resulting from the Max–TEF-1 interaction has to be contributed entirely by the much larger protein, TEF-1. In human TEF-1, at least three transactivation domains have been identified (32). These regions are also responsible for TEF-1’s autointerference squelching activity in HeLa cells, and these results in the dominant negative phenotype of TEF-1 overexpression (34, 64). Recently, several studies have shown that TEF-1 interacts with TBP (13, 27, 34); this association inhibits the ability of TBP to bind to the TATA element (34), which in turn might lead to inhibition of transcription due to disruption of preinitiation complex formation. This observation was supported by evidence that the transcriptional repression effect of TEF-1 could be reversed by overexpression of TBP (34), thus suggesting that the squelching effect of TEF-1, as observed in HeLa and other cells, is likely to be the result of the binding of TEF-1 to TBP. In these circumstances, Max might act as a transcription coactivator by binding to TEF-1, which might antagonize TEF-1-mediated inhibition of TBP-TATA complex formation and thus lead to transcription induction. Similarly, a yeast transcription factor, AD1, disrupts TBP-TATA complex formation; however, this effect is blocked by prior association of the TBP complex with TF-IIA (2). Another possibility is that the binding of TEF-1 to Max leads to the formation of a stable complex with the EM motif and that this association can lead to the recruitment of another activator protein which bridges the trans-acting factors at the EM motif to the component of the preinitiation complex, leading to transcriptional activation. Our results support this concept, as besides the TEF-1–Max complex, C1, a higher-order protein complex, C2, also occurred at the EM site. The discrimination between these possibilities and definition of the precise mechanism of the transactivation function of the TEF-1–Max complex must await identification of the amino acid domains of two proteins that mediate their physical association.

As more transcription factors have been characterized, it has become clear that the existence of networks of factors interacting at a single cis regulatory element is a common feature. The data showing physical interaction between TEF-1 and Max, together with the observed functional interaction, indicate that this form of regulation may occur on the promoter containing binding sites for either one or both of these transcription factors. In such an interaction, the cell specificity of the complex might be determined in part by TEF-1, because several tissue-restricted isoforms of TEF-1 have been identified whereas Max has been shown to be widely expressed. Whether TEF-1 could also interact with other bHLH proteins remains to be determined.

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