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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 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 (CANNTG) 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).

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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-IIc 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 (18). 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 acetylcholine 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 cDNA fragment from pBSMax (1) was isolated by *Nco*I and *Sac*I digestion and subcloned into the *Nco*I and *Sac*I sites of pGEX-KG. The last 92 amino acids from the carboxy terminus of the c-Myc protein were amplified by PCR, digested with *Eco*RI and *Hin*dIII, and subcloned into the *Eco*RI and *Hin*dIII sites of the pGEX-KG vector. The full-length TEF-1 protein was amplified from pBS-TEF-1 (59) by PCR, digested with *Xba*I and *Xho*I, and subcloned into the *Xba*I and *Xho*I 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 pSV-Max 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.EM.CAT contains a fragment consisting of bp −156 to bp +30 of the α-MHC gene linked immediately upstream to the chloramphenicol acetyltransferase (CAT) reporter gene in the promoterless pGCATC vector. 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 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-^{32}P]ATP (56). The analytical binding reaction was carried out in a total volume of 25 μl containing approximately 10,000 cpm (0.1 to 0.5 ng) of the labeled DNA probe, 2 to 5 μg of the nuclear extract, and 1 μg of poly(dI-dC) (Sigma) as a nonspecific competitor. The binding buffer consisted of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM dithiothreitol (DTT), 0.3 mM $MgCl_2$, 8% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride. After incubation at room temperature for 20 min, the reaction mixtures were loaded on 5% native polyacrylamide gels and electrophoresis was carried out at 150 V in a 0.5× Tris-borate-EDTA buffer in the cold room, unless indicated otherwise in the figure legends. For competition and antibody experiments, unlabeled competitor DNA or the antibody was preincubated with the nuclear extract in the reaction buffer at room temperature for 15 to 20 min prior to addition of the labeled DNA probe. The Max antibody used in this 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 11 amino acids of the human TEF-1 protein (a gift from P. 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 Laemmli protein sample buffer supplemented with 0.2 M NaCl. The complex was eluted from the acrylamide by crushing the gel with a glass stirring rod and incubating the slurry at 37°C for 2 h and then at 95°C for 2 min and then spun through a Centrex spin filter (Schleicher and Schuell). The filtrate was resolved on a sodium dodecylsulfate (SDS)–12% 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 pGST-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 0.6 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 of the GST fusion proteins, cells were harvested and the fusion proteins were isolated as follows. Cells were pelleted at 4,000 × *g* and 4°C, 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* and 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 precipitation 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 neonatal rat heart. Five micrograms of GST or GST-CTMyc 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,

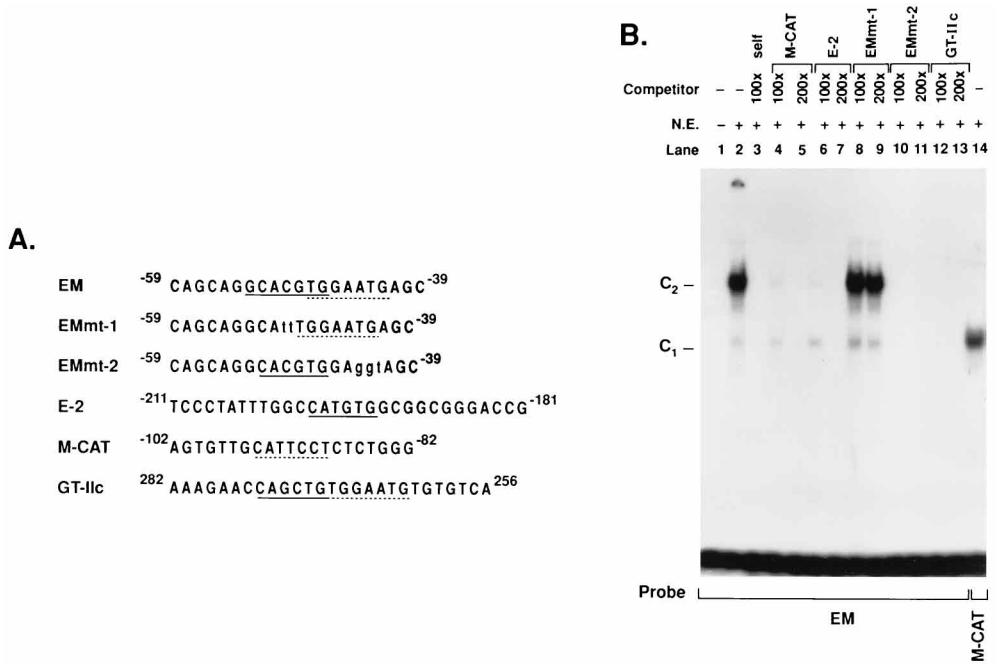

FIG. 1. Both E-box and M-CAT binding proteins interact with the α-MHC EM motif. (A) Sense-strand sequence of double-stranded oligonucleotides used in the experiment. The position of each E box is underlined, and each M-CAT element is indicated by a broken line. Nucleotides in lowercase letters indicate mutation from the wild-type oligonucleotide. (B) The end-labeled α-MHC EM (lanes 1 to 13) and cTNT M-CAT (lane 14) probes were incubated with 4 μg of neonatal rat heart nuclear extract (N.E.), and DNA-protein complex formation was analyzed on a 5% polyacrylamide gel. The molar excess of unlabeled competitor oligonucleotides is given above each lane. Competitor oligonucleotides are as follows: self, same as the probe; M-CAT, cTNT M-CAT; E-2, cardiac α-actin E-2; GT-IIc, SV40 GT-IIc; EM, wild-type α-MHC EM; EMmt-1, mutation in the E-box site of the EM motif; and EMmt-2, mutation in the M-CAT site of the EM motif. C_1 and C_2 are the fast- and slow-migrating complexes generated by the EM probe, respectively.

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 [^{35}S]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, ^{35}S-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 $MgCl_2$, 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 Laemlli 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-cAMP-Max, was generated to synthesize the GST-cAMP-Max fusion protein. A double-stranded oligonucleotide that encodes two cAMP-dependent protein kinase A (PK-A) sites was introduced into the *Bam*HI and *Eco*RI sites of pGEX-KG to create the pGEX-cAMP plasmid. Subsequently, an *Nco*I-*Sac*I fragment (containing full-length Max cDNA) was isolated from pBS-Max (1) and subcloned into the *Nco*I and *Sac*I sites of the plasmid GEX-cAMP to direct expression of GST-cAMP-Max. The GST-cAMP-Max fusion protein was overexpressed in DH5α 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 2×10^5 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 $MgCl_2$, 1 mM DTT, and 0.05% NP-40). The NC blot was incubated with the labeled GST-cAMP-Max 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 2×10^6 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 antimyosin antibody (19). 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% CO_2. 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/plate 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 GAC GAT GAC AAA ATT GAG CCC AGC AGC TGG AGC-3′) contained a *Kpn*I site and had an optimum ATG, a Flag sequence, and sequence complementary to the 5′ end of TEF-1. The 3′ primer (5′-GGG CTC GAG TCA GTC CTT CAC AAG CCT GTA GAT-3′) contained sequence complementary to the 3′ end of TEF-1 that included the native stop codon of TEF-1 and a *Xho*I restriction site. The PCR product was digested with *Kpn*I and *Xho*I and subcloned into *Kpn*I and *Xho*I sites of the pCMV-5 vector. Cos7 cells, at a density of 10^6/plate, were transfected with the expression plasmids pCMV.Flag-TEF-1 and pSV.Max by the lipofectin 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

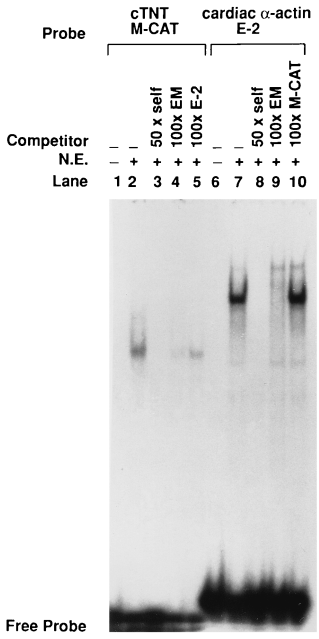

FIG. 2. Factors binding to the cTNT M-CAT and cardiac α-actin E-2 elements are recognized by the α-MHC EM motif. EMSAs were performed with different end-labeled probes and neonatal rat heart nuclear extract (N.E.). The molar excess of unlabeled competitor oligonucleotides (as described in the legend to Fig. 1) is given above each lane.

conjugated to agarose beads (1.8 mg of antibody/ml) in a total volume of 1 ml for 1 h with continuous rocking at 4°C. The beads were pelleted and washed five times in the lysis buffer, and the resultant proteins were suspended in 2× Laemlli buffer and subsequently subjected to Western blot analysis with Max antisera.

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 and the α-MHC EM oligonucleotide as a labeled probe. As shown in Fig. 1, two specific DNA-protein complexes of different mobilities and intensities were detected, a slowly migrating major complex, C_2, and a faster-migrating minor complex, C_1. The C_1 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 5 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, an oligonucleotide corresponding to the E-2 element of avian cardiac α-actin was used as a competitor. This E-2 element contains a canonical E-box binding site (CATGTG) that has been shown before to be necessary for the expression of the cardiac α-actin gene in cardiac muscle cells (46). As shown in Fig. 1B, an excess of the E-2 oligonucleotide also competed effectively for both the C_1 and C_2 DNA-protein complexes of the α-MHC EM motif (lanes 6 and 7). Furthermore, when an inverse experiment was performed in which oligonucleotides corresponding to cardiac α-actin E-2 and cTNT M-CAT sequences were used as labeled probes, the excess of the α-MHC EM oligonucleotide competed effectively for the specific complex formed by these probes (Fig. 2, lanes 4 and 9). Thus, these results suggest that at least two proteins, one related to TEF-1 and the other related to an E-box binding protein, are part of the C_1 and C_2 complexes generated by the α-MHC EM motif.

To distinguish between these two DNA-protein interactions, 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).

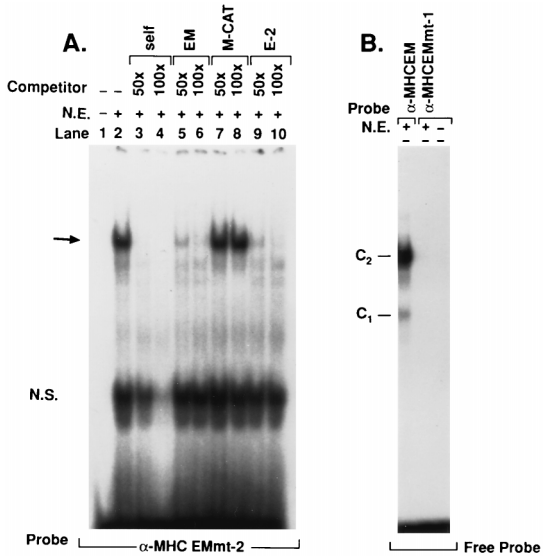

FIG. 3. The EM-mt-2 oligonucleotide retains protein binding ability, but the EM-mt-1 oligonucleotide does not. (A) The end-labeled probes were incubated with neonatal rat heart nuclear extract (N.E.), and the formation of the complex was analyzed on a 5% polyacrylamide gel. The molar excess of unlabeled competitor oligonucleotides (as described in the legend to Fig. 1) is given above each lane. The arrow indicates the specific DNA-protein complex. N.S., nonspecific. In panel (B) Wild-type α-MHC EM probe was used as a control.

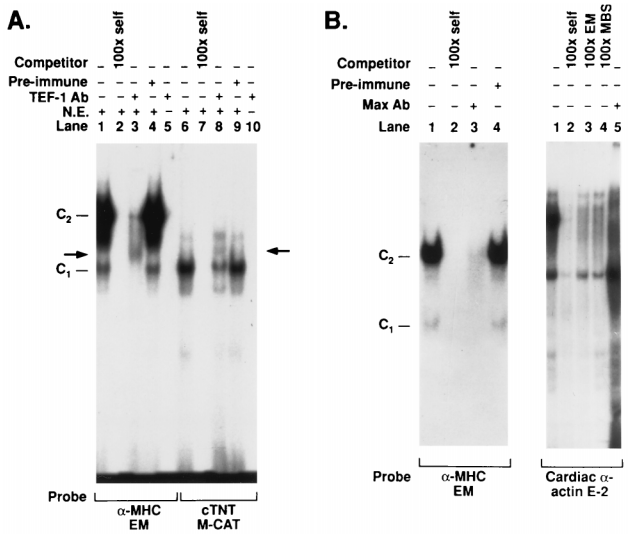

FIG. 4. TEF-1 and Max proteins are part of both the C_1 and C_2 complexes of the α-MHC EM probe. The cTNT M-CAT and cardiac α-actin E-2 templates were utilized as positive controls for binding of TEF-1 and Max proteins, respectively. In the EMSA reaction, cardiac nuclear extract (N.E.) was preincubated 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 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 C_1-EM and C_2-EM complexes, and a small portion of the C_1-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 GST–TEF-1 fusion protein–EM complex formation, and results similar to those achieved with the nuclear extract were obtained (data not shown). These data confirm our previous results, obtained by Western blotting and UV cross-linking analyses, documenting that a 52- to 54-kDa TEF-1-related protein is present in the EM-protein complex (30).

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 C_1 and C_2 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 C_1 and C_2 complexes of the α-MHC EM motif. Antibodies against c-Myc and myogenin were tested as well in this experiment, and neither affected the formation of a complex with the EM oligonucleotide (data not shown).

Max associates with the M-CAT–TEF-1 complex even in the absence of an E-box binding site. The inability of the anti-Max antibodies to induce specific supershifting could be due to the distinct nature of the endogenous Max-oligonucleotide complex. Similarly, a lack of specific supershifting by anti-Max antisera was observed before by others analyzing the Myc/Max binding site (MBS) of the ornithine decarboxylase gene (51). To confirm that Max is indeed a part of the DNA-protein complex obtained with the α-MHC EM and cardiac α-actin E-2 oligonucleotides, we next performed Western blot analysis. In this experiment, we decided to use the cTNT M-CAT oligonucleotide as a negative control because it lacks an E-box binding site (Fig. 1A) and had previously failed to compete for the E-2–protein complex (Fig. 2, lane 10). Surprisingly, as shown in Fig. 5A, a band with a molecular mass similar to that of Max (21 to 22 kDa) was observed not only for the EM (both C_1 and C_2) and E-2 complexes but also for the M-CAT–protein complex. The detection of a Max-related protein from the M-CAT complex was totally unexpected, as this complex does not contain an apparent Max (E-box) 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-

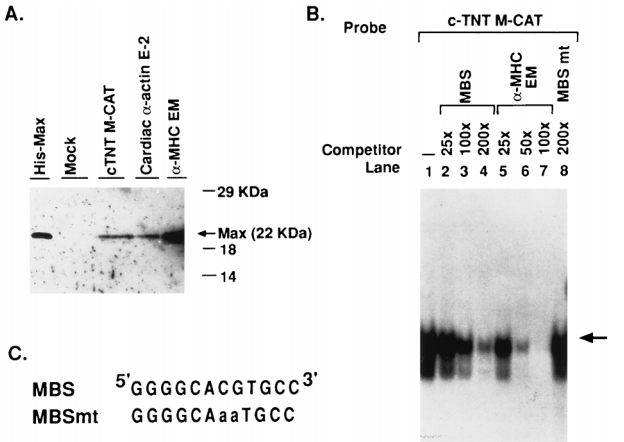

FIG. 5. Max is a part of the cardiac α-actin E-2, α-MHC EM, and cTNT M-CAT–protein complexes. (A) Western blot analysis. DNA-protein complexes obtained with unlabeled probes by EMSA were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (as described in Materials and Methods). 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 C_2 complex; however, Max was also detected in the C_1 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).

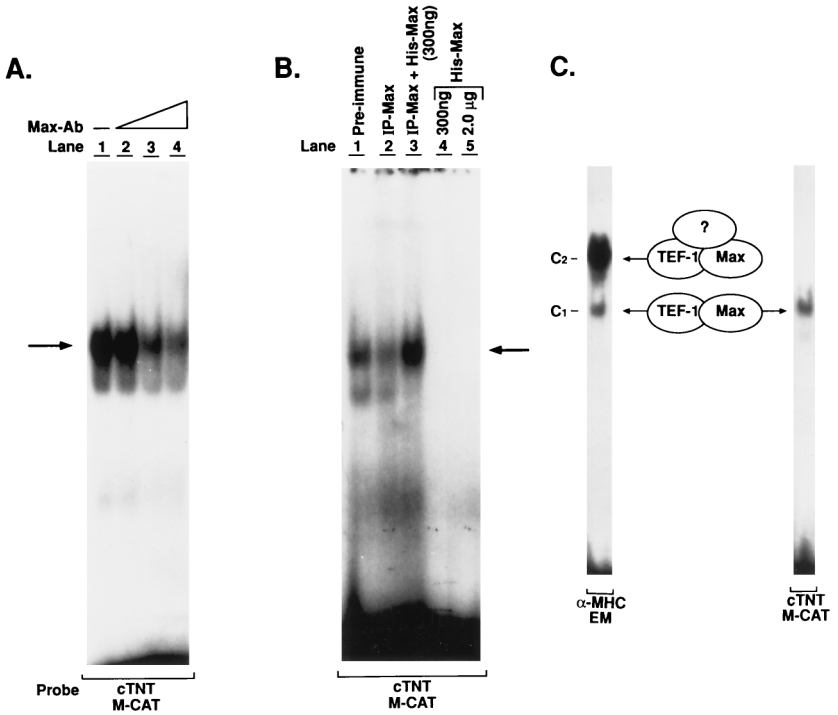

FIG. 6. Max is part of the cTNT M-CAT complex even when Max by itself does not bind to the DNA template. (A) In the EMSA reaction, cardiac nuclear extract was preincubated with increasing concentrations of Max antibody (Max-Ab) (2, 4, and 8 μl in lanes 2, 3, and 4, respectively). (B) Lanes 2 and 3: Max was immunoprecipitated by Max antibody, and the EMSA was performed with Max-depleted nuclear extract (IP-Max); in lane 3, the EMSA reaction mixture also contained 300 ng of His-Max. Lane 1: cardiac nuclear extract cleared with preimmune serum was used for EMSA. Lanes 4 and 5: EMSA was performed with 300 ng and 2.0 μg of His-Max protein, respectively. Bovine serum albumin was included in the EMSA reaction mixtures so that the protein content in each lane was kept constant. The arrow indicates the position of the specific DNA-protein complex. (C) Summary of the complexes, showing that the cTNT M-CAT complex is identical to the C_1 complex of the α-MHC EM motif; both contain TEF-1 as well as Max proteins. The C_2 complex of the EM motif is a multiprotein complex that includes TEF-1, Max, and another, unidentified factor(s) that may associate with them.

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 C_1 complex of the EM motif whereas the C_2 complex is a higher-order protein association that includes proteins present in the C_1 complex as well as another, unidentified factor(s) that may interact with it (Fig. 6C).

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 after 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

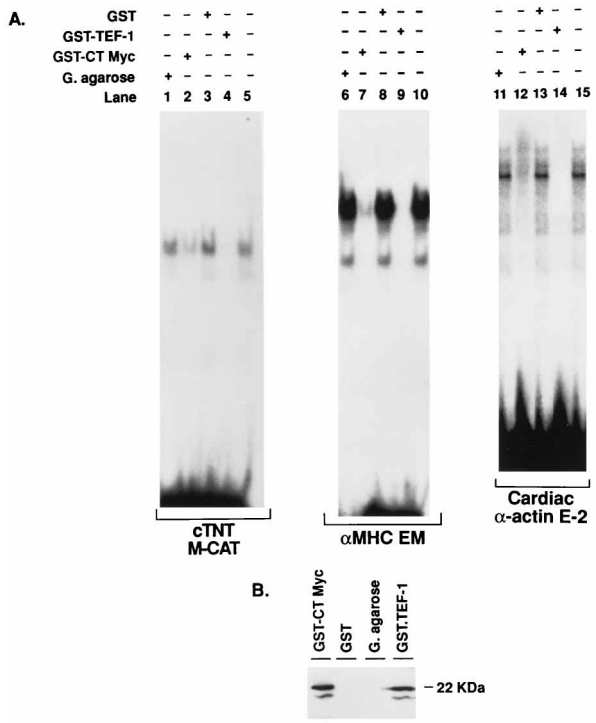

FIG. 7. Affinity precipitation of Max from nuclear extract by GST-CTMyc and GST–TEF-1. (A) Forty micrograms of cardiac nuclear extract diluted fivefold in 1× binding buffer was incubated with 5 μg of glutathione-agarose beads bound with GST fusion proteins (GST-CTMyc or GST–TEF-1), GST, or beads alone (G. agarose) for 3 h at 4°C. Glutathione-agarose beads were pelleted, and EMSA was performed with equal volumes of supernatant from each tube. (B) Pelleted beads were subjected to Western blot analysis with anti-Max antibody.

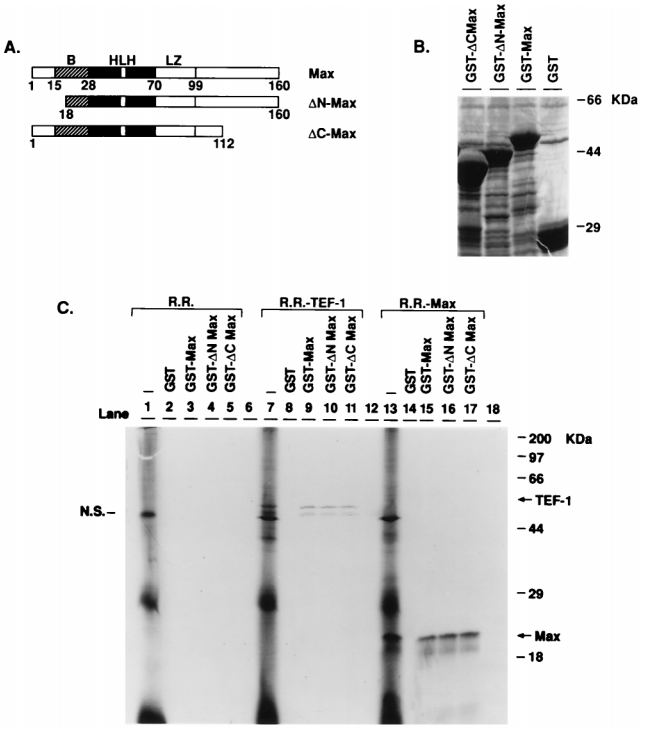

FIG. 9. Interaction of TEF-1 with GST-Max truncated polypeptides. (A) Schematic diagram indicating different regions in the full-length Max, and of the ΔN-Max and ΔC-Max truncated peptides. (B) Coomassie blue staining of bacterially synthesized GST and GST-Max fusion peptides. (C) In the rabbit reticulocyte lysate, pBS-TEF-1 (R.R.-TEF-1) was transcribed and translated with [^{35}S]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.

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 [^{35}S]methionine-labeled Max was retained on the beads that contained GST–TEF-1 (Fig. 8, lane 3). Reciprocally, [^{35}S]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

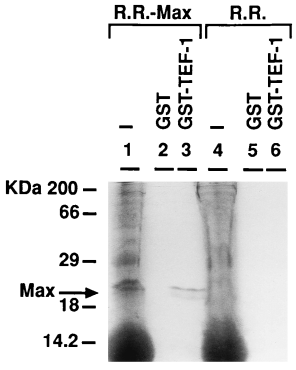

FIG. 8. Interaction of GST–TEF-1 with in vitro-translated Max. In the rabbit reticulocyte lysate, pBS-Max (R.R.-Max) was transcribed and translated with [^{35}S]methionine. The labeled proteins were incubated with glutathione-agarose beads bound to GST or GST–TEF-1. Proteins bound to beads were analyzed on SDS-polyacrylamide gels in the lanes indicated. A parallel experiment was performed with plasmidless R.R., which served as a negative control.

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 ^{35}S-labeled TEF-1. As a positive control, interaction of these GST-Max mutants with in vitro-synthesized full-length Max was also tested, and, as expected, each showed binding to the full-length Max (Fig. 9C, lanes 15 to 17). These findings confirm the binding of Max to TEF-1 and indicate that the region of Max contributing to this interaction must consist of between 18 and 112 amino acids, comprising the bHLH-LZ region of the protein. Experiments involving other mutations in the Max and TEF-1 proteins are in progress to determine the exact amino acids of the two proteins that are responsible for their association.

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 ^{32}P-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

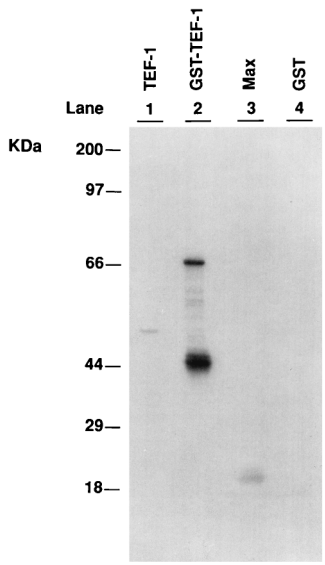

FIG. 10. Interaction between TEF-1 and Max as determined by far-Western analysis. Fifty-nanogram quantities of different proteins, as designated above the lanes, were resolved by SDS-PAGE, transferred to an NC membrane, and hybridized with ^{32}P-labeled GST-cAMP-Max fusion protein as described in Materials and Methods.

with TEF-1 even when the protein is immobilized on the NC membrane.

Finally, to demonstrate in vivo TEF-1–Max interaction, cells were cotransfected with expression plasmids encoding either full-length TEF-1 or Max proteins. The TEF-1 expression plasmid used in this experiment contained a Flag peptide coding sequence linked immediately upstream of the TEF-1 cDNA in order to synthesize a Flag-tagged TEF-1 protein. After transfection, a cell lysate was prepared. It was examined for the expression of Flag–TEF-1 and Max proteins (Fig. 11A and B) and then incubated with the anti-Flag affinity gel. To test whether Max was coimmunoprecipitated with the Flag–TEF-1 protein, these beads were washed repeatedly and subjected to Western blot analysis with the anti-Max antibody. As shown in Fig. 11C, a ~22-kDa Max protein was pulled down from cells transfected with both Max and TEF-1 expression plasmids but not from other cells that did not receive the Flag–TEF-1 plasmid. These results confirm TEF-1–Max interaction in vivo.

Cooperative transcriptional activation by Max and TEF-1. 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$^-$ 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.

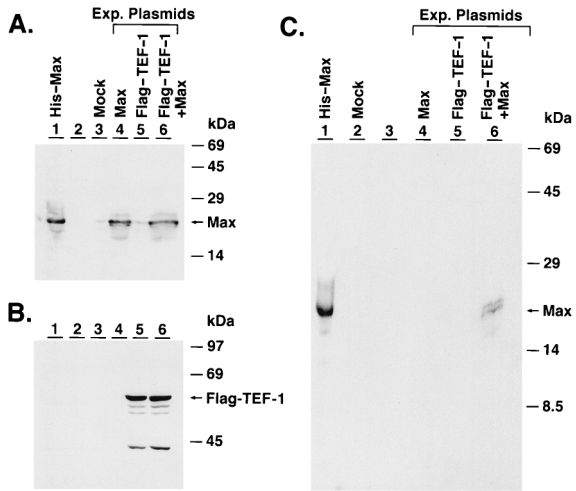

FIG. 11. Characterization of in vivo TEF-1–Max interaction as determined by coimmunoprecipitation of proteins. Cells were transfected with expression (Exp.) plasmid pSV.Max and/or pCMV.Flag-TEF-1 together. (A and B) Expression of ectopically expressed proteins as verified by Western blot analysis with anti-Max antibody (A) or anti-Flag antibody (B). In panel B, lanes 1 to 6 are the same as in panel A. (C) Coimmunoprecipitation of Max with TEF-1. The whole-cell extract was incubated with the anti-Flag antibody-conjugated agarose beads. The beads were pelleted, washed, and subjected to Western blot analysis with anti-Max antibody. Fifty nanograms of His-Max was used as a positive control.

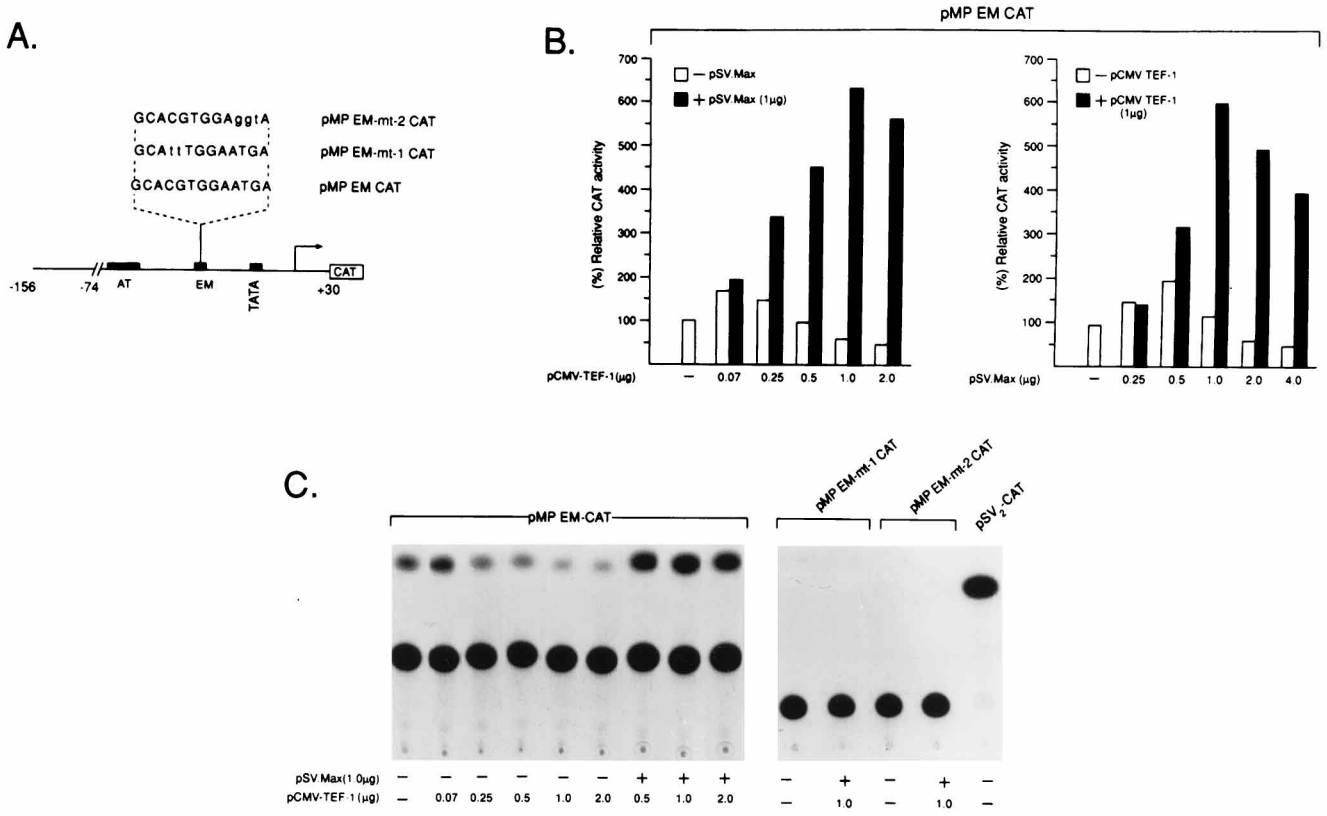

FIG. 12. Cooperative transactivation by TEF-1 and Max. Primary cultures of cardiac myocytes were transfected with various recombinant plasmids, and transcriptional activation or repression was assayed by measurement of CAT activity from 5 μg of the promoter-reporter construct. (A) Configuration of different α-MHC–CAT reporter constructs used in the study. AT, AT-rich element. (B) Left panel: effect of different concentrations of TEF-1 plasmid alone (open bars) and together with 1 μg of Max plasmid (black bars) on pMP.EM.CAT expression; right panel: an inverse experiment with increasing concentrations of the Max plasmid (open bars) alone and together with 1 μg of TEF-1 plasmid. (C) Representative CAT assays normalized with β-galactosidase activity measured in the same cell lysate. pSV_2CAT was used as a positive control for transfection efficiency.

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

TABLE 1. Adjacent E-box (CACGTG) and M-CAT (CATTCCT) sequences in the promoter regions of different muscle-specific and SV40 genes

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

[a] 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.

[b] The E-box elements of the different genes are aligned for comparison and are shown in boldface letters, whereas M-CAT elements are underlined.

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 template; 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 template. 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 heterotypic 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 to 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

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 undetectable, 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 that 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, C_1, a higher-order protein complex, C_2, 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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REFERENCES

1. **Amin, C., A. J. Wagner, and N. Hay.** 1993. Sequence-specific transcriptional activation by Myc and repression by Max. Mol. Cell. Biol. **13:**383–390.
2. **Auble, D. T., and S. Hahn.** 1993. An ATP-dependent inhibitor to TBP binding to DNA. Genes Dev. **7:**844–856.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology, vol. 1. Green Publishing Associates and John Wiley & Sons, New York, N.Y.
4. **Ayer, D. E., L. Kretzner, and R. N. Eisenman.** 1993. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72:**211–222.
5. **Azakie, A., S. B. Larkin, I. K. G. Farrance, G. Grenningloh, and C. P. Ordahl.** 1996. D-TEF-1, a novel member of the transcriptional enhancer factor-1 (TEF-1) multigene family. J. Biol. Chem. **271:**8260–8265.
6. **Berberich, C., I. Durr, M. Koenen, and V. Witzemann.** 1993. Two adjacent E-box elements and an M-CAT box are involved in the muscle specific regulation of the rat acetylcholine receptor β subunit gene. Eur. J. Biochem. **216:**395–404.
7. **Berberich, S. J., and M. D. Cole.** 1992. Casein kinase II inhibits the DNA binding activity of Max homodimers but not Myc/Max heterodimers. Genes Dev. **6:**166–176.
8. **Berberich, S. J., N. Hyde-DeRuyscher, P. Espenshade, and M. D. Cole.** 1992. Max encodes a sequence specific DNA binding protein and is not regulated by serum growth factors. Oncogene **4:**775–779.
9. **Berger, L. C., D. B. Smith, I. Davidson, J.-J. Hwang, E. Fanning, and A. G. Wildeman.** 1996. Interaction between T antigen and TEA domain of the factor TEF-1 derepresses simian virus 40 late promoter in vitro: identification of T-antigen domains important for transcriptional control. J. Virol. **70:**1203–1212.
10. **Blackwood, E. M., and R. N. Eisenman.** 1991. Max: A helix-loop-helix zipper protein that forms a sequence specific DNA binding complex with Myc. Science **251:**1211–1217.
11. **Blatt, C., and M. L. DePamphilis.** 1993. Striking homology between mouse and human transcription enhancer factor-1 (TEF-1). Nucleic Acids Res. **21:**747–748.
12. **Bouvagnet, P. F., E. E. Strehler, G. E. White, M.-A. Strehler-Page, B. Nadal-Ginard, and V. Mahdavi.** 1987. Multiple positive and negative 5′ regulatory elements control the cell-type-specific expression of the embryonic skeletal myosin heavy-chain gene. Mol. Cell. Biol. **7:**4377–4389.
13. **Brou, C., S. Chaudhary, I. Davidson, Y. Lutz, G. Wu, J. M. Egly, L. Tora, and P. Chambon.** 1993. Distinct TF-IID complexes mediate the effect of different transcriptional activators. EMBO J. **12:**489–499.
14. **Burglin, T. R.** 1991. The TEA domain: a novel highly conserved DNA binding motif. Cell **66:**11–12.
15. **Campbell, S., M. Inamdar, V. Rodrigues, K. V. Raghavan, M. Palazzolo, and A. Chovnick.** 1992. The scalloped gene encodes a novel evolutionarily conserved transcription factor required for sensory organ differentiation in *Drosophila*. Genes Dev. **6:**367–379.
16. **Chaudhary, S., C. Brou, M.-E. Valentin, N. Burton, L. Tora, P. Chambon, and I. Davidson.** 1994. A cell-specific factor represses stimulation of transcription in vitro by transcriptional enhancer factor 1. Mol. Cell. Biol. **14:** 5290–5299.
17. **Chaudhary, S., L. Tora, and I. Davidson.** 1995. Characterization of a HeLa cell factor which negatively regulates transcriptional activation in vitro by transcriptional enhancer factor-1 (TEF-1). J. Biol. Chem. **270:**3631–3637.
18. **Chen, Z., G. A. Friedrich, and P. Soriano.** 1994. Transcriptional enhancer factor-1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev. **8:**2293–2301.
19. **Clark, W. A., R. A. Chizzonite, A. W. Everett, M. Rabinowitz, and R. Zak.** 1982. Species correlation between cardiac isomyosins: a comparison of electrophoretic and immunological properties. J. Biol. Chem. **257:**5449–5454.
20. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell **68:**597–612.
21. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
22. **Ferrance, I. K. G., J. H. Mar, and C. P. Ordahl.** 1992. M-CAT binding factor is related to SV40 enhancer binding factor TEF-1. J. Biol. Chem. **267:**17234–17240.
23. **Flink, I. L., J. G. Edwards, J. J. Bahl, C. C. Liew, M. Sole, and E. Morkin.** 1992. Characterization of a strong positive cis-acting element of the human β-myosin heavy chain gene in fetal rat heart cells. J. Biol. Chem. **267:**9917–9924.
24. **Fortuin, D., K. I. Kariya, A. F. R. Stewart, D. G. Rokosh, C. S. Long, and P. C. Simpson.** 1995. Transcriptional enhancer factor-1 (TEF-1) isoform mRNAs are expressed differentially in rat heart and other tissues. J. Cell. Biochem., p. 118. (Keystone Symposium proceedings.)
25. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. **9:**5022–5033.
26. **Grichnik, J., D. Bergsma, and R. Schwartz.** 1986. Tissue-restricted and stage specific transcription is maintained within 411 nucleotides flanking the 5′ end of the chicken skeletal α-actin gene. Nucleic Acids Res. **14:**1683–1701.
27. **Gruda, M. C., J. M. Zabolotny, J. H. Xiao, I. Davidson, and J. C. Alwine.** 1993. Transcriptional activation by simian virus 40 large T antigen: interactions with multiple components of the transcription complex. Mol. Cell. Biol. **13:**961–969.
28. **Grueneberg, D. A., S. Natesan, C. Alexander, and M. Gilman.** 1992. Human and *Drosophila* homeodomain proteins that enhance the DNA binding activity of serum-response factor. Science **257:**1089–1095.
29. **Guan, K. L., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in *E. coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. Anal. Biochem. **192:**262–267.
30. **Gupta, M. P., M. Gupta, and R. Zak.** 1994. An E-box/M-CAT hybrid motif and cognate binding protein(s) regulate the basal muscle specific and cAMP inducible expression of the rat cardiac α-myosin heavy chain gene. J. Biol. Chem. **269:**29677–29687.
31. **Heikinheimo, M., J. M. Scandrett, and D. B. Wilson.** 1994. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. Dev. Biol. **164:**361–373.
32. **Hwang, J. J., P. Chambon, and I. Davidson.** 1993. Characterization of transcriptional activation function and DNA binding domain of transcriptional enhancer factor-1. EMBO J. **12:**2337–2348.
33. **Jia, H. T., H. J. Tsay, and J. Schmidt.** 1992. Analysis of binding and activating functions of the chick muscle acetylcholine receptor γ-subunit upstream sequence. Cell. Mol. Neurobiol. **12:**241–258.
34. **Jiang, S. W., and N. L. Eberhardt.** 1996. TEF-1 transrepression in BeWo cells is mediated through interaction with TATA binding protein TBP. J. Biol. Chem. **271:**9510–9518.
35. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
36. **Kato, G. J., W. M. F. Lee, L. Chen, and C. V. Dang.** 1992. Max-functional domains and interaction with c-Myc. Genes Dev. **6:**81–92.
37. **Larkin, S. B., I. K. G. Farrance, and C. P. Ordahl.** 1996. Flanking sequences modulate the cell specificity of M-CAT elements. Mol. Cell. Biol. **16:**3742–3755.
38. **Litvin, J., M. O. Montgomery, D. J. Goldhamer, C. P. Emerson, Jr., and D. M. Bader.** 1993. Identification of DNA-binding proteins in the developing heart. Dev. Biol. **156:**409–417.
39. **Mar, J. H., and C. P. Ordahl.** 1990. M-CAT binding factor, a novel *trans*-acting factor governing muscle-specific transcription. Mol. Cell. Biol. **10:** 4271–4283.
40. **Melin, F., M. Miranda, N. Montreau, M. L. DePamphilis, and D. Blangy.** 1993. Transcription enhancer factor-1 (TEF-1) DNA binding sites can specifically enhance gene expression at the beginning of mouse development. EMBO J. **12:**4657–4666.
41. **Miner, J. H., J. B. Miller, and B. J. Bold.** 1992. Skeletal muscle phenotypes initiated by ectopic MyoD in transgenic mouse hearts. Development **114:** 853–860.
42. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. **6:**2125–2136.
43. **Molkentin, J. D., B. L. Black, J. F. Martin, and E. N. Olson.** 1995. Cooperative activation of muscle gene expression by MEF-2 and myogenic bHLH proteins. Cell **83:**1125–1136.
44. **Molkentin, J. D., R. S. Brogan, S. M. Jobe, and B. E. Markham.** 1993. Expression of the α-myosin heavy chain gene in the heart is regulated in part by an E-box dependent mechanism. J. Biol. Chem. **268:**2602–2609.
45. **Molkentin, J. D., D. V. Kalvakolanu, and B. E. Markham.** 1994. Transcription factor GATA-4 regulates cardiac muscle-specific expression of the α-myosin heavy-chain gene. Mol. Cell. Biol. **14:**4947–4957.
46. **Moss, G. B., T. C. McQuinn, and R. T. Schwartz.** 1994. The avian cardiac α-actin promoter is regulated through a pair of complex elements composed of E-boxes and SREs that bind both positive- and negative-acting factors. J. Biol. Chem. **269:**12731–12740.
47. **Navankasattusas, S., M. Sawadogo, M. van Bilsen, C. V. Dang, and K. R. Chien.** 1994. The basic helix-loop-helix protein upstream stimulating factor regulates the cardiac ventricular myosin light-chain 2 gene via independent *cis* regulatory elements. Mol. Cell. Biol. **14:**7331–7339.
48. **Ojama, K., A. M. Samarel, and I. Klein.** 1996. Identification of a contractile-responsive element in the cardiac α-myosin heavy chain gene. J. Biol. Chem. **270:**31276–31281.
49. **Olson, E. N.** 1993. Regulation of muscle gene transcription by the MyoD family. The heart of the matter. Circ. Res. **72:**1–6.
50. **Ordahl, C. P.** 1992. Developmental regulation of sarcomeric gene expression. Curr. Top. Dev. Biol. **26:**145–168.
51. **Pena, A., D. Reddy, S. Wu, N. J. Hickok, E. P. Reddy, G. Yumet, D. R.**

Soprano, and K. J. Soprano. 1993. Regulation of human ornithine decarboxylase expression by the c-Myc-Max protein complex. J. Biol. Chem. **268:** 27277–27285.
52. **Piette, J., A. Klarsfeld, and J. P. Changeux.** 1989. Interaction of nuclear factors with the upstream region of the α-subunit gene of chicken muscle acetylcholine receptor: variations with muscle differentiation and denervation. EMBO J. **8:**687–694.
53. **Prody, C. A., and J. P. Merlie.** 1992. The 5′ flanking region of the mouse muscle nicotinic acetylcholine receptor β-subunit gene promotes expression in cultured muscle cells and is activated by MRF-4, myogenin and myoD. Nucleic Acids Res. **20:**2367–2372.
54. **Rosenthal, N.** 1987. Identification of regulatory elements of cloned genes with transcriptional assays. Methods Enzymol. **152:**704–720.
55. **Ruoqian-Shen, S. K. Goswami, E. Mascareno, A. Kumar, and M. A. Q. Siddiqui.** 1991. Tissue-specific transcription of the cardiac myosin light-chain 2 gene is regulated by an upstream repressor element. Mol. Cell. Biol. **11:**1676–1685.
56. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
57. **Sartorelli, V. N., A. Hong, N. H. Bishopric, and L. Kedes.** 1992. Myocardial activation of the human cardiac α-actin promoter by helix-loop-helix proteins. Proc. Natl. Acad. Sci. USA **89:**4047–4051.
58. **Seidman, C. E., D. W. Wong, J. A. Jarcho, K. D. Bloch, and J. G. Seidman.** 1988. *cis*-Acting sequences that modulate atrial natriuretic factor gene expression. Proc. Natl. Acad. Sci. USA **85:**4104–4108.
59. **Shimizu, N., G. Smith, and S. Izumo.** 1993. Both a ubiquitous factor, m-TEF-1, and a distinct muscle specific factor bind to the M-CAT motif of the myosin heavy chain β gene. Nucleic Acids Res. **21:**4103–4110.
60. **Sternberg, E. A., G. Spizz, W. M. Perry, D. Vizard, T. Weil, and E. N. Olson.** 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. **8:**2896–2909.
61. **Stewart, A. F. R., S. B. Larkin, I. K. G. Farrance, J. H. Mar, D. E. Hall, and C. P. Ordahl.** 1994. Muscle enriched TEF-1 isoforms bind M-CAT elements from muscle specific promoters and differentially activates transcription. J. Biol. Chem. **269:**3147–3150.
62. **Wagner, A. J., M. M. L. Beau, M. O. Diaz, and N. Hay.** 1992. Expression regulation and chromosomal localization of the Max gene. Proc. Natl. Acad. Sci. USA **89:**3111–3115.
63. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks and thresholds. Cell **75:**1241–1244.
64. **Xiao, J. H., I. Davidson, H. Matthes, J. M. Garnier, and P. Chambon.** 1991. Cloning, expression and transcriptional properties of the human TEF-1. Cell **65:**551–568.
65. **Xiao, J. H., I. Davidson, D. Ferrandon, R. Rosales, M. Vigneron, M. Macchi, F. Ruffenach, and P. Chambon.** 1987. One cell-specific and three ubiquitous nuclear proteins bind in vitro to overlapping motifs in the domain B-1 of the SV40 enhancer. EMBO J. **6:**3005–3013.
66. **Yasunami, M., K. Suzuki, T. Houtani, T. Sugimoto, and H. Ohkubo.** 1995. Molecular characterization of cDNA encoding a novel protein related to transcriptional enhancer factor-1 from neural precursor cells. J. Biol. Chem. **270:**18649–18654.
67. **Yu, Y. T., R. E. Beribert, L. B. Smoot, Y. Lee, V. Mahdavi, and B. Nadal-Ginard.** 1992. Human myocyte specific enhancer factor II comprises a group of tissue restricted MADS box transcription factors. Genes Dev. **6:**1783–1796.
68. **Zervos, A., J. Gyuris, and R. Brent.** 1993. Mxi, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.