

cDNA Cloning and Characterization of Murine Transcriptional Enhancer Factor-1-related Protein 1, a Transcription Factor That Binds to the M-CAT Motif*

(Received for publication, August 16, 1995, and in revised form, October 16, 1995)

Courtland E. Yockey^{‡§}, Gillian Smith[‡], Seigo Izumo^{‡¶}, and Noriko Shimizu^{‡§||}

From the [‡]Molecular Medicine Division, Beth Israel Hospital and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215 and [§]Department of Developmental Biology and Anatomy, University of South Carolina, School of Medicine, Columbia, South Carolina 29208

The M-CAT motif is a cis-regulatory DNA sequence that is essential for muscle-specific transcription of several genes. Previously, we had shown that both muscle-specific (A1) and ubiquitous (A2) factors bind to an essential M-CAT motif in the myosin heavy chain β gene and that the ubiquitous factor is transcriptional enhancer factor (TEF)-1. Here we report the isolation of mouse cDNAs encoding two forms (a and b) of a TEF-1-related protein, TEFR1. The TEFR1a cDNA encodes a 427-amino acid protein. The coding region of TEFR1b is identical to 1a in both nucleotide and predicted amino acid sequence except for the absence of 43 amino acids downstream of the TEA DNA-binding domain. Three TEFR1 transcripts (~7, ~3.5, and ~2 kilobase pairs) are enriched in differentiated skeletal muscle (myotubes) relative to undifferentiated skeletal muscle (myoblasts) and non-muscle cells in culture. *In situ* hybridization analysis indicated that TEFR1 transcripts are enriched in the skeletal muscle lineage during mouse embryogenesis. Transient expression of fusion proteins of TEFR1 and the yeast GAL4 DNA-binding domain in cell lines activated the expression of chloramphenicol acetyltransferase (CAT) reporter constructs containing GAL4 binding sites, indicating that TEFR1 contains an activation domain. An anti-TEFR1 polyclonal antibody supershifted the muscle-specific M-CAT-A1 factor complex in gel mobility shift assays, suggesting that TEFR1 is a major component of this complex. Our results suggest that TEFR1 might play a role in the embryonic development of skeletal muscle in the mouse.

Commitment and subsequent differentiation of skeletal muscle involves a cascade of transcription factors that act through several different cis-regulatory elements. The best characterized of these elements are the E-box and A/T-rich motifs, which interact with the MyoD/bHLH (1) and MEF-2/MADS (2) families of transcription factors, respectively. Members of the MyoD

family have been shown to be critical to the development of skeletal muscle (1, 3). Members of the MEF-2 family are involved in both cardiac and skeletal muscle development (2, 4–6).

In addition to elements that are involved in muscle-lineage determination, other cis-regulatory elements that have been shown to be involved in the expression of muscle-specific genes include the serum response element, SP-1 element, and M-CAT motif (6–10). The M-CAT motif was initially identified as a cis-regulatory element in the cardiac troponin T promoter (6). The M-CAT motif has subsequently been shown to be required for muscle-specific expression from the myosin heavy chain β (9–11) and α -skeletal actin (7) promoters *in vitro*, as well as the myosin heavy chain β (12) and α (13) promoters *in vivo*.

Two mammalian M-CAT-binding proteins have been identified, transcriptional enhancer factor-1 (TEF-1)¹ (17, 20, 22) and embryonic TEA domain-containing factor (ETF) (23). ETF transcripts are strictly limited in their distribution during embryogenesis, appearing predominately in the hindbrain at embryonic day 10 by *in situ* hybridization analysis. Little is known about ETF function except that it shares binding-site specificity with TEF-1. TEF-1 is a cellular transcriptional activator that was originally identified as a viral cis-regulatory element-binding protein that interacts with the GT-IIC and Sph motifs (14–17). The GT-IIC motif is almost identical to the M-CAT motif, which TEF-1 has been shown to bind as well (7, 13, 18–21). TEF-1 transcripts are present in most adult tissues, and are particularly abundant in the kidney, skeletal muscle, heart, and lung. During embryogenesis, TEF-1 appears to be expressed ubiquitously (24). TEF-1 contains a TEA DNA-binding domain (25) and an activation function that involves the cooperation of at least three regions of the protein (acidic and proline-rich and C-terminal regions) (26). TEF-1 also interacts with cell-specific co-factors, some of which are required for activation (27), others of which block activation (28). A transgenic mouse strain containing a null mutation of the TEF-1 gene shows homozygous embryonic lethality around embryonic day 11 due to a heart malformation characterized by an abnormally thin ventricle wall (24). However, the skeletal muscle lineage appears to be unaffected in these mice, which suggests that TEF-1 is not essential for the early stages of skeletal muscle development.

We have previously shown that two M-CAT (A-element)

* This work was supported in part by National Institutes of Health Grant AR41893 and the American Heart Association, Massachusetts Affiliate, Grant 13-519-912. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) L26343 (TEFR1a) and L26344 (TEFR1b).

¶ Present address: Division of Cardiology, University of Michigan Medical Center, Ann Arbor, MI 48109.

|| To whom correspondence should be addressed: Dept. of Developmental Biology and Anatomy, University of South Carolina, School of Medicine, Columbia, SC 29208. Tel.: 803-733-3215; Fax: 803-733-1533; E-mail: shimizu@cdmsserver.med.sc.edu.

¹ The abbreviations used are: TEF-1, transcriptional enhancer factor-1; CAT, chloramphenicol acetyltransferase; aa, amino acid(s); ETF, embryonic TEA domain-containing factor; GST, glutathione S-transferase; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; mTEF1, mouse TEF-1; cTEF1, chicken TEF-1; TEFR1, TEF-1-related protein 1; bp, base pair(s); kb, kilobase pair(s).

binding factors, A1 and A2, could be distinguished by gel mobility shift assays using nuclear extracts from differentiated skeletal muscle cells in culture (myotubes) (29). The A2 factor appears to be ubiquitous, while the A1 factor is seen only in myotubes. We cloned the mouse homolog of TEF-1 (mTEF-1) and showed that it is one component of the A2 factor (20). However, the identity of the A1 factor remained unknown. Because the A1 and A2 factors had similar DNA-binding properties (29), we concluded that these factors might be closely related. By using TEF-1 cDNA as a probe, we isolated cDNAs for TEFR1, an M-CAT-binding transcription factor that bears a close resemblance to TEF-1. We present evidence here suggesting that TEFR1 is a candidate for the muscle-specific A1 factor. We also show that TEFR1 transcripts are enriched in the skeletal myogenic lineage during mouse embryogenesis.

MATERIALS AND METHODS

Preparation of Sol8 Myotube cDNA Library—Poly(A)⁺ RNA from Sol8 myotubes was prepared using oligo(dT)-cellulose (Invitrogen). The Sol8 myotube Uni-ZAP cDNA library was prepared using the ZAP-cDNA synthesis kit (Stratagene). Bluescript SK(−) phagemids containing cDNA inserts between *Eco*RI and *Xho*I sites were excised from the λZAP clones using the ExAssist helper phage.

Cloning of TEF-1-related Transcription Factors—An ~800-bp cDNA clone encoding a TEF-1-related protein was isolated by screening an adult mouse cardiac λZAPII cDNA library (Stratagene) using human TEF-1 cDNA (kindly provided by Dr. Pierre Chambon) (17) as a probe under reduced stringency conditions as described previously (20). Using the TEF-1-related cDNA as a probe, we further screened the Sol8 myotube cDNA library. Prehybridization was performed at 42 °C in a solution containing 50% formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 M Tris-HCl, pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed at 42 °C in the same solution with the denatured probe, which was labeled by the random oligo priming method using a kit (Boehringer Mannheim) according to the manufacturer's instructions. The filter was washed in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at room temperature, followed by washing in 0.1 × SSC and 0.1% SDS at 65 °C.

DNA Sequencing and Analysis—Nested deletions of plasmids TEFR1a and 1b were created using an ExoIII/mung bean nuclease deletion kit (Stratagene). Double-stranded DNA sequencing was performed on both strands using the Sequenase kit (U. S. Biochemical Corp.) and/or using the Taq DyeDeoxy Terminator Cycle Sequencing kit and DNA Sequencer (Applied Biosystems). All computer analyses of nucleotide (nt) and amino acid (aa) sequences were done using the GCG package of sequence analysis tools (30).

Northern Blot Analysis—Total RNA from cells was extracted as previously described (31). Total RNA from different adult mouse tissues was extracted as described elsewhere (32). Poly(A)⁺ RNA from Sol8 myotubes and 3T3 cells was prepared using oligo(dT)-cellulose (Invitrogen). Either total RNA (30 μg) or poly(A)⁺ RNA (3 μg) was electrophoresed on 1% agarose-1.1 M formaldehyde gels, and transferred to nylon filters. All probes were prepared by random oligo priming using a kit (Boehringer Mannheim). Prehybridization and hybridization were performed at 42 °C in the same solution as described for cDNA cloning (see above). The filters were washed in 2 × SSC and 0.1% SDS at room temperature, followed by washing in 0.1 × SSC and 0.1% SDS at 60 °C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Amplification—RT-PCR amplifications were conducted using a kit (Perkin-Elmer) according to the manufacturer's instructions with modifications. Reverse transcription was primed using random hexamers from 500 ng of total RNA. One-fifth of this reaction mixture was used for PCR. The PCR was conducted in an automated thermal cycler (Perkin-Elmer) for either 24 cycles (see Fig. 3B) or 35 cycles (Fig. 3C). The TEFR1-specific amplimers corresponded to nt 103–124 of TEFR1a (sense) and nt 703–722 of TEFR1a (antisense). PCR products were electrophoresed on 1.5% agarose gels and transferred to nylon membranes. A sense-strand oligonucleotide (nt 575–595 of TEFR1a) was used as a Southern blotting probe. Control amplifications were performed using either mouse β₂-microglobulin amplimers (sense: 5'-TGC TAT CCA GAA AAC CCC TC-3'; antisense: 5'-ATG CTG ATC ACA TGT CTC GAT-3'; Southern blotting probe [sense]: 5'-CGC CTC ACA TTG AAA TCC AA-3') (33) for 16 cycles (Fig. 3B), or mouse β-actin amplimers (Stratagene) for 22

cycles (Fig. 3C).

In Situ Probe Preparation—A PCR product containing the first non-coding exon of the mouse α-cardiac actin gene, nt −26 to +113 (34), was prepared from Sol8 genomic DNA and cloned into *Hind*III-*Bam*HI sites of Bluescript SK(+). The TEFR1 probe template corresponds to the complete coding region, nt 110–1393 of TEFR1a (Fig. 1A), generated by PCR from the original cDNA clone and ligated into the *Bam*HI-*Xba*I sites of Bluescript SK(+). Radioactive RNA probes (riboprobes) were prepared using either T3 or T7 RNA polymerase (Stratagene) and ³⁵S-UTP (DuPont NEN) as described elsewhere (35). TEFR1 probes were alkaline hydrolyzed as previously described (36) to an average length of 150 nt, which was verified by denaturing polyacrylamide gel electrophoresis (PAGE).

In Situ Hybridization—Embryos were obtained from timed-pregnant CD-1 mice (Charles River) and fixed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C. Dehydration, embedding, and section preparation were conducted as described elsewhere (35). Prehybridization treatment of tissue sections, hybridization, and posthybridization treatment were conducted as previously described (34), with the omission of proteinase K digestion. Riboprobes were applied directly to tissue sections at an activity of approximately 75,000 dpm/μl. Emulsion autoradiography using Kodak NTB-2 emulsion, counterstaining with toluidine blue, and subsequent mounting were conducted as previously described (35). Slides dipped in emulsion were developed after exposure for 10 days in all cases.

In Vitro Transcription and Translation—Plasmids mTEF-1 (20) and TEFR1 (1a and 1b) were linearized with *Nde*I and *Xho*I, respectively. The linearized plasmids were transcribed using either T7 or T3 RNA polymerase in the presence of m7G(5')ppp(5')G in addition to NTPs. One microgram of RNA product was translated with 20 μl of rabbit reticulocyte lysate (Stratagene) in the presence of [³⁵S]methionine (ICN).

Preparation of Glutathione S-transferase (GST) Fusion Proteins—GST-fusion protein plasmids, pGEX-TEFR (aa 1–24), pGEX-TEFR (aa 1–38), pGEX-mTEF (aa 1–29), and pGEX-mTEF (aa 1–430), were constructed by ligating either TEFR1a or mTEF-1 cDNA fragments produced by PCR into either *Bam*HI-*Eco*RI or *Bam*HI-*Sma*I sites of pGEX-2T (Pharmacia Biotech Inc.). Both 5' and 3' junctions were sequenced to verify that the fragments were inserted in-frame. Expression of GST-fusion proteins (see Fig. 7A) in *Escherichia coli* was induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The fusion proteins were purified using glutathione-Sepharose 4B beads (Pharmacia) as described by the manufacturer.

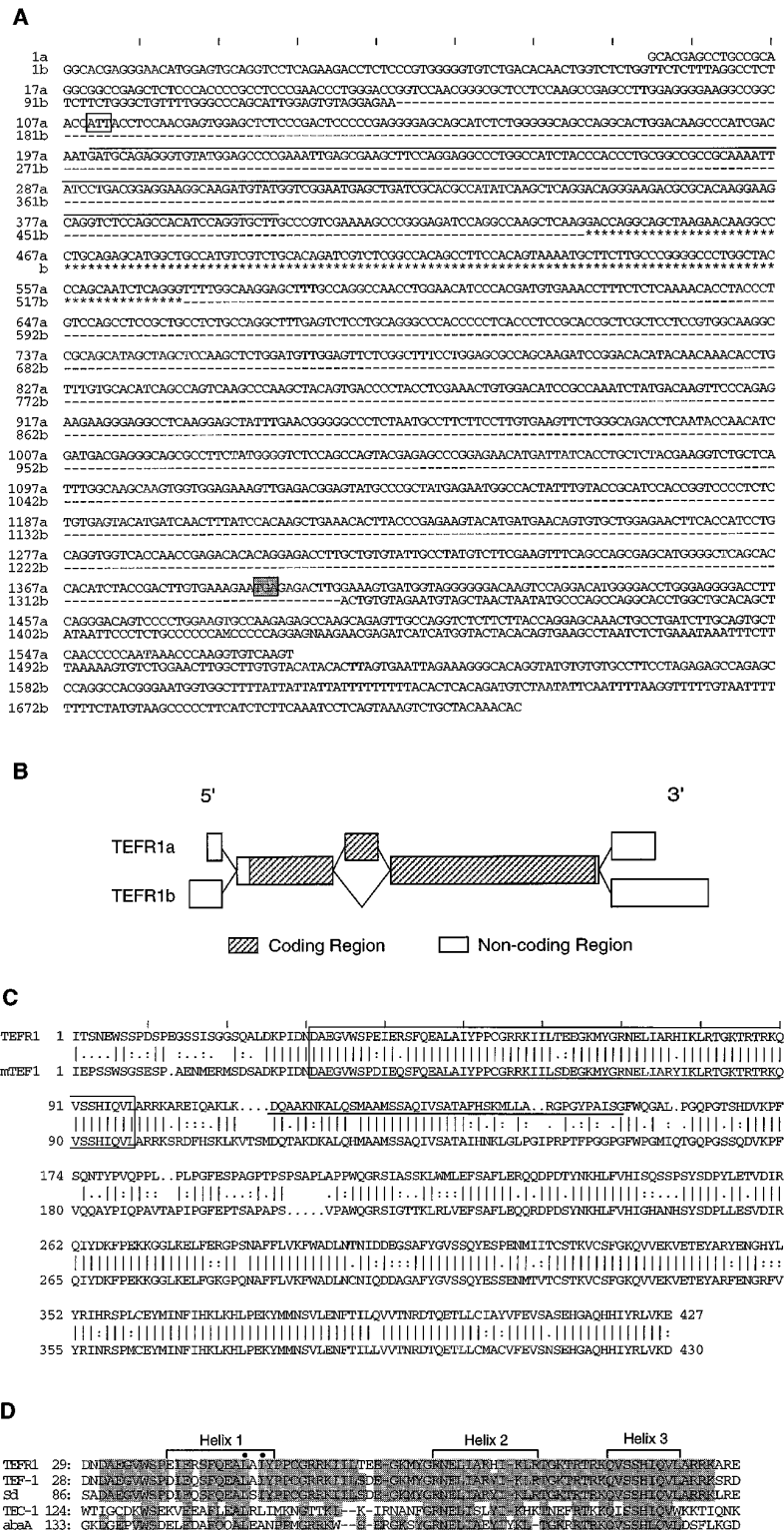
Preparation of Antibody—Anti-TEFR1 polyclonal antibody was produced by immunization of chickens with GST-TEFR(1–38) fusion protein. All immunizations, collections of serum and IgY purifications from eggs were performed by East Acres Biologicals, Inc. (Southbridge, MA).

Immunoprecipitation—Five microliters of *in vitro* transcription/translation products of TEFR1b and mTEF-1 cDNA clones (see above) in 200 μl of radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% Nonidet P-40) were precleared by incubating for 1 h at 4 °C with rocking in the presence of 20 μg of preimmune chicken IgY, 15 μg of rabbit anti-chicken IgY and 30 μl of 50% protein A-Sepharose (Pharmacia) slurry. After spinning for 2 min at 500 × g, the supernatants were incubated for 1 h on ice with 20 μg of anti-TEFR1 antibody and 15 μg of rabbit anti-chicken IgY in the absence or presence of an equimolar amount of different competitors. They were further incubated for 1 h at 4 °C with rocking in the presence of 30 μl of 50% protein A-Sepharose slurry. The Sepharose beads were washed four times with radioimmune precipitation buffer, resuspended in SDS sample buffer (37), and incubated in a boiling water bath for 5 min. Proteins were separated on an SDS-PAGE gel.

Gel Mobility Shift Assay—Preparation of nuclear extracts and binding reactions for gel mobility shift assays were carried out as described elsewhere (29). For supershift experiments, nuclear extracts were preincubated with either preimmune or anti-TEFR1 antibody at room temperature for 30 min in the absence or presence of an equimolar amount of different competitors. Reaction mixtures were further incubated with labeled DNA probe and poly(dI-dC) at room temperature for 15 min. Native PAGE gels were run at 150 V in a 0.5 × TBE buffer (35). The sequence of the sense strand of oligo A (M-CAT) is 5'-CAG GCA GTG GAA TGC GAG GAG-3' (29). This oligo was annealed to a complementary oligo to form the double-stranded probe.

Construction of GAL4-fusion Plasmids—The GAL4/TEFR1-fusion plasmids, GAL4-TEFR 1–7 (see Fig. 9), were constructed by replacement of the *Bam*HI-*Xba*I fragment of pBS(RSV)-GAL4(1–147)-E2F(Δ1–367) (kindly provided by Dr. Erick K. Flemington) with TEFR1 fragments produced by PCR. Both 5' and 3' junctions were sequenced to

FIG. 1. **cDNA and predicted amino acid sequence analysis of TEFR1.** A, cDNA sequence of TEFR1. The sense strand is shown. The sequences of TEFR1a (*upper*) and 1b (*lower*) are numbered separately from the 5' termini of respective cDNA clones. *Dashes* within the TEFR1b sequence indicate identity to TEFR1a. Nucleotides which do not appear in TEFR1b are indicated by *asterisks*. An *open box* indicates the position of the probable initiation codon (ATT). A *shaded box* indicates the position of the termination codon (TGA). Nucleotides that correspond to the TEA DNA-binding domain are *overlined*. B, structures of TEFR1a and 1b cDNAs. *Open* and *hatched boxes* indicate the noncoding and coding regions, respectively. C, predicted amino acid sequence of TEFR1a (*upper*) and comparison with the predicted mTEF-1 sequence (*lower*) (20). Between the two sequences, *vertical bars* indicate identity, and *colons* and *single dots* indicate strong and weak similarity, respectively. *Dots* within each sequence represent gaps which were introduced to maximize homology. The TEA DNA-binding domain is *boxed*. The 43-aa region absent from TEFR1b is *underlined*. D, comparison of the amino acid sequences of TEA DNA-binding domains of mouse TEFR1, human TEF-1 (17), *Drosophila* *scalloped* (*Sd*) (55), *Saccharomyces cerevisiae* TEC-1 (56), and *Aspergillus nidulans* *abaA* (57). The TEA domains of human, mouse (20), and chicken (21) TEF-1 as well as ETF (23) are identical in amino acid sequence. Conserved amino acids are *shaded*. *Dashes* indicate gaps which were introduced to maximize homology. The *numbers* to the left of each line indicate the position of the first amino acid shown in each protein. The locations of predicted helices are indicated above the TEFR1 sequence. The locations of amino acids mutated in this study are indicated by *black dots*.



verify that TEFR1 fragments were inserted in-frame. The mutants GAL4-TEFR 1_M and GAL4-TEFR 2_M were constructed by replacement of the *Bam*HI-*Not*I fragments of GAL4-TEFR 1 and GAL4-TEFR 2, respectively, with the mutated fragments produced by PCR. The positions of mutated amino acids are marked in Figs. 1D and 9.

Cell Culture—Mouse skeletal muscle (Sol8 and C2C12), mouse fibroblast (Swiss 3T3), and human cervical carcinoma (HeLa) cell lines were maintained in growth medium containing Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with either 20% (Sol8 and C2C12) or 10% (3T3 and HeLa) fetal bovine serum. Differentiation of Sol8 and C2C12 myoblasts was induced by exposure of confluent cultures to differentiation medium containing Dulbecco's modified Eagle's medium

and 10% horse serum.

Transfection and Enzyme Assay—DNA transfection was performed by the calcium phosphate precipitation method (38) as described previously (29). CAT and β -galactosidase activity in the cell extracts were assayed as described previously (39, 40).

RESULTS

TEFR1a and 1b Are TEF-1-related Transcription Factors—We isolated TEF-1-related cDNA clones by screening a mouse cardiac cDNA library using human TEF-1 cDNA as a probe under low stringency conditions as described previously

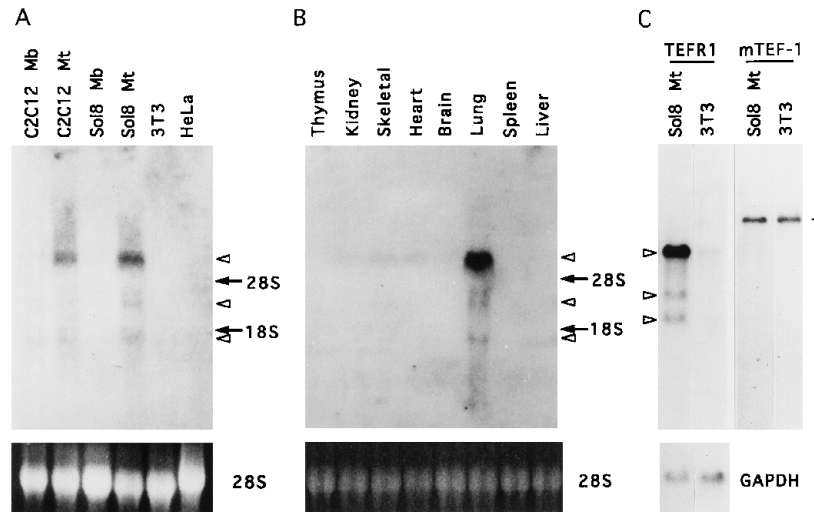


FIG. 2. Northern blot analysis of TEFR1 transcripts. A and B, total RNAs were isolated from either cell lines (A) or adult mouse tissues (B). Thirty micrograms of total RNAs were separated on a 1% agarose gel and transferred to a nylon filter. The full-length coding region of the TEFR1a cDNA clone was used as a probe. The positions of 28 S and 18 S ribosomal RNAs are indicated. The positions of mRNAs (~ 7 , ~ 3.5 , and ~ 2 kb) hybridized with the TEFR1a probe are indicated by open arrowheads. Ethidium bromide staining of 28 S ribosomal RNA prior to transfer of the gel is shown at the bottom. Mb, myoblasts; Mt, myotubes. C, poly(A)⁺ RNAs were prepared from Sol8 myotubes (Mt) and 3T3 fibroblasts. Three micrograms of poly(A)⁺ RNAs were separated on a 0.8% agarose gel and transferred to a nylon filter. The filter was hybridized with a probe containing the full-length coding region of the TEFR1a cDNA (left). The same filter was sequentially rehybridized with a labeled *Eco*RI fragment of mTEF1 cDNA (right) and a labeled GAPDH cDNA (bottom) (20). The positions of mRNAs hybridized with the TEFR1a probe and the mTEF1 probe (~ 12 kb) are indicated by open and solid arrowheads, respectively.

(20). We then screened a Sol8 myotube cDNA library using one of the cardiac cDNA clones, TEFR1 (~ 0.8 kb), as a probe under high stringency conditions. About 20 clones were isolated from an initial screening of 5×10^5 plaques. Two different clones (TEFR1a and 1b) containing full-length coding regions were selected by restriction mapping and partial DNA sequencing for further analysis. The TEFR1a and 1b clones were ~ 1.6 and ~ 1.7 kb in length, respectively. The DNA sequences of these clones are shown in Fig. 1A.

The open reading frame of the TEFR1a cDNA encodes a 427 aa protein containing a TEA DNA-binding domain (aa 31–98; Fig. 1, A–C). The initiation codon used by TEFR1 appears to be AUU (Ile) at nt 110 (see “Discussion”). The open reading frame of the TEFR1b cDNA encodes a 384-aa protein, which is identical to TEFR1a except that 43 aa downstream of the TEA domain are absent from TEFR1b (Fig. 1, B and C). The full coding nucleotide sequence of TEFR1b is identical to that of TEFR1a, except that a 129-nt region (nt 443–571 in TEFR1a) is absent from TEFR1b (Fig. 1, A and B). The noncoding regions of the two cDNAs are divergent, except for sequences proximal to the start and stop codons, which are identical (Fig. 1, A and B). Comparison of the TEFR1 and mTEF-1 (20) predicted amino acid sequences revealed 76% overall identity and 93% identity within the TEA domain (Fig. 1C). The TEA DNA-binding domain consists of three putative α -helices (25) and is highly conserved throughout evolution. Except for TEFR1, all other vertebrate TEA proteins share 100% amino acid identity within the TEA domain (Fig. 1D) (23).

TEFR1 Transcripts Are Enriched in Embryonic Skeletal Muscle and Adult Lung—We examined the expression of TEFR1 transcripts in various cell lines and tissues by Northern blot analysis using a probe containing the full coding region of the TEFR1a cDNA clone (Fig. 2). A major ~ 7 -kb transcript and minor ~ 3.5 - and ~ 2 -kb transcripts were observed in differentiated skeletal muscle cells (Sol8 and C2C12 myotubes), but not in undifferentiated skeletal muscle cells (Sol8 and C2C12 myoblasts) or non-muscle cells (3T3 and HeLa) (Fig. 2A). In adult tissues, TEFR1 transcripts are abundant in lung, present at a low level in kidney, skeletal muscle, and heart, and undetect-

able in thymus, brain, spleen, and liver (Fig. 2B). In poly(A)⁺ RNA, TEFR1 transcripts are present at a low level in non-muscle (3T3) cells and are abundant in differentiated skeletal muscle cells (Sol8 myotubes) (Fig. 2C). In contrast to the expression pattern of TEFR1 transcripts, a ~ 12 -kb mTEF-1 transcript was found at a similar level in both Sol8 myotubes and 3T3 cells, as previously observed (Fig. 2C) (20).

RT-PCR was used to distinguish between TEFR1a and 1b transcripts (Fig. 3). Both TEFR1a (620 bp) and 1b (491 bp) PCR products were seen in Sol8 and C2C12 myotubes (Fig. 3B). Both TEFR1a and 1b were also observed in adult lung, skeletal muscle, heart, and kidney (Fig. 3C). A very small amount of TEFR1 was seen in Sol8 and C2C12 myoblasts and non-muscle cells (3T3) (Fig. 3B). TEFR1b has consistently appeared to be more abundant than 1a in all tissues and cell lines tested under the RT-PCR conditions used here, except in 3T3 cells. In 3T3 cells, TEFR1a has consistently appeared to be more abundant than 1b.

We also examined the expression of TEFR1 transcripts during mouse development by *in situ* hybridization using anti-sense riboprobes made by *in vitro* transcription of the full coding region of the TEFR1a cDNA. TEFR1 transcripts are enriched in the myotome at embryonic day 9 (Fig. 4, A–E), co-localized with α -cardiac actin (Fig. 4, C and F). At embryonic day 14.5, TEFR1 transcripts are enriched in embryonic skeletal muscle (Fig. 5B), co-localized with α -cardiac actin (Fig. 5A). α -Cardiac actin has been shown to be a marker for embryonic striated muscle (34). *In situ* hybridization using sense riboprobes showed background levels of hybridization over tissue sections (Fig. 5, C and D).

TEFR1 Binds to the M-CAT Motif (A Element) *In Vitro*—We examined whether TEFR1a and 1b could bind to the M-CAT motif in a gel mobility shift assay. *In vitro* transcription/translation products of mTEF-1, TEFR1a, and 1b cDNAs were analyzed by SDS-PAGE (Fig. 6A). The mTEF-1 product migrated as a major ~ 53 -kDa band as described previously (20). The major TEFR1a product was slightly smaller (~ 52 kDa) than mTEF-1, and the major TEFR1b product was smaller (~ 50 kDa) than TEFR1a. Using these *in vitro* transcription/transla-

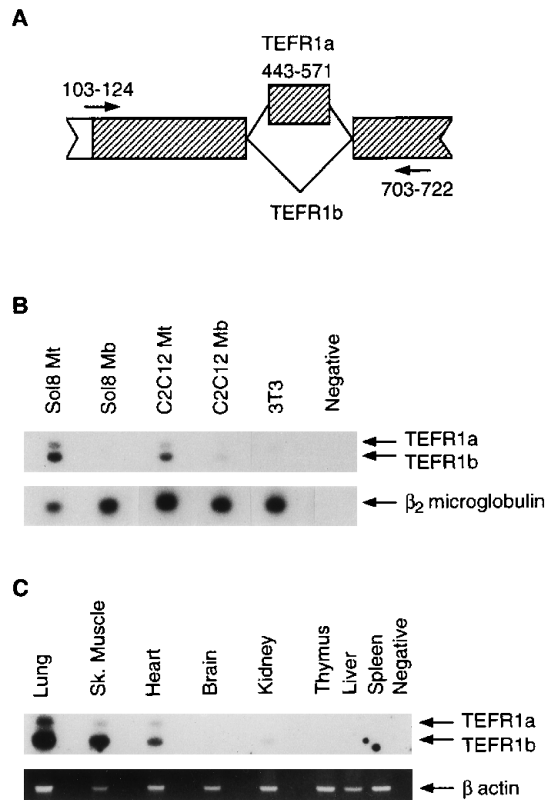


FIG. 3. RT-PCR analysis of TEFR1 transcripts. A, a portion of the TEFR1 cDNAs from Fig. 1B. Primers used for PCR (sense nt 103–124 of TEFR1a and antisense nt 703–722) are indicated by arrows. B, total RNAs from mouse skeletal muscle (Sol8 and C2C12) and fibroblast (Swiss 3T3) cell lines were subjected to RT-PCR using specific amplimers for either TEFR1 (24 cycles) or β_2 -microglobulin (16 cycles). PCR products were separated by agarose gel electrophoresis and Southern blotted. The 620- and 491-bp bands correspond to TEFR1a- and 1b-specific PCR products, respectively. The negative control is in the absence of RNA. Mb, myoblasts; Mt, myotubes. C, total RNAs from adult mouse tissues were subjected to RT-PCR using TEFR1-specific (35 cycles) and β -actin-specific (22 cycles) amplimers. PCR products were separated by agarose gel electrophoresis and Southern blotted. Ethidium bromide staining of β -actin products is shown at the bottom. The negative control is in the absence of RNA.

tion products and end-labeled oligo A containing the M-CAT motif from the rabbit myosin heavy chain β promoter as a probe (29), we performed a gel mobility shift assay (Fig. 6B). TEFR1a and mTEF-1 formed specific protein-DNA complexes with the same mobility as that of the ubiquitous M-CAT-A2 factor-complex, while TEFR1b formed a specific complex with the same mobility as that of the muscle-specific M-CAT-A1 factor complex. The specificity of TEFR1 binding was examined in competition experiments with unlabeled oligo A and MutaA (29) as a competitor (Fig. 6C). Unlabeled oligo A competed for complex formation between labeled oligo A and TEFR (1a and 1b). In contrast, unlabeled oligo MutaA, which contains a mutated M-CAT motif, had no effect on complex formation.

Muscle-specific A1 Factor Is Antigenically Related to TEFR1 and Distinct from mTEF-1—We generated a polyclonal antibody against a GST-fusion protein containing the amino-terminal 38 aa of TEFR1 (GST-TEFR(1–38)) (Fig. 7A). To determine the specificity of this antibody, we conducted immunoprecipitations of *in vitro* transcription/translation products of the TEFR1b and mTEF-1 cDNAs (Fig. 7B) in the presence of one of several GST-fusion protein competitors (Fig. 7A). In the absence of competitor (–) or the presence of GST as a competitor (GST), both TEFR1b (lanes 2 and 3) and mTEF-1 (lanes 8 and 9) were immunoprecipitated. In the presence of the immunogen

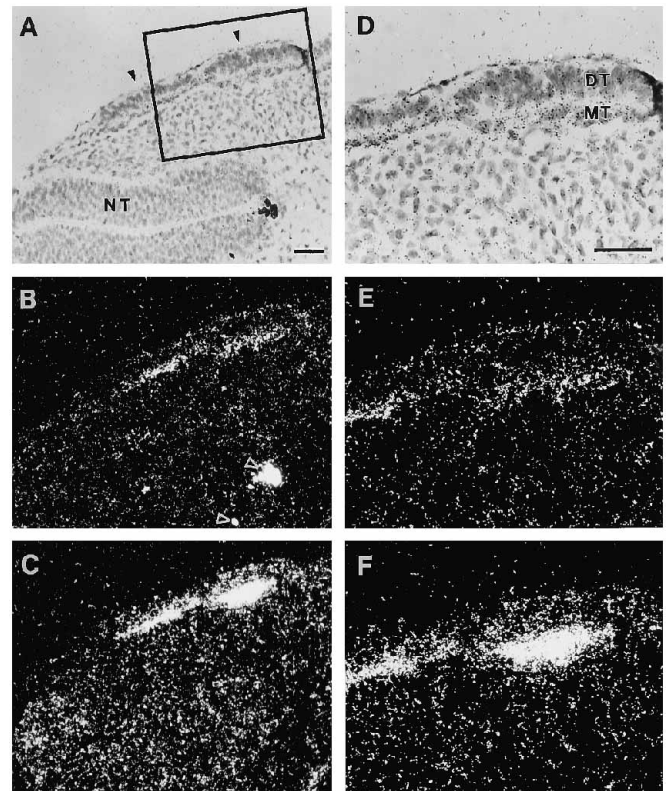


FIG. 4. *In situ* hybridization to serial sections of an embryonic day 9 mouse embryo, showing somites. Antisense riboprobes complementary to either TEFR1 (A, B, D, and E) or α -cardiac actin (C and F) transcripts were used. B, C, E, and F are views under dark-field optics. A and D are bright-field views of B and E, respectively. D, E, and F are higher magnification views of A, B, and C, respectively; the box in A indicates the magnified area. Solid arrowheads in A indicate somites. Arrowheads in B indicate artifactual debris. DT, dermatome; MT, myotome; NT, neural tube. Bar, 50 μ m.

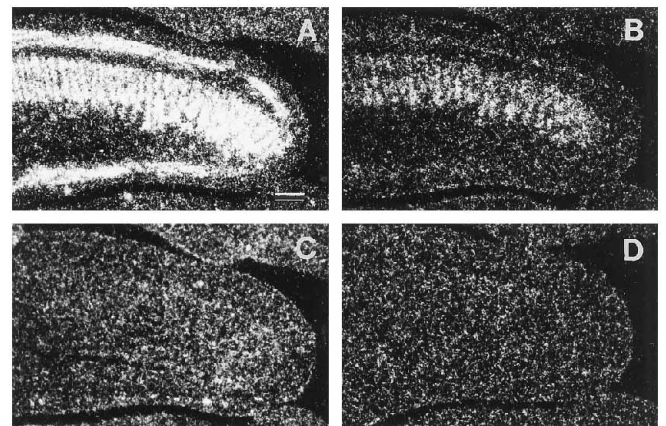


FIG. 5. *In situ* hybridization to parasagittal sections of an embryonic day 14.5 mouse embryo, showing tongue. All views are under dark-field optics. The following riboprobes were used: α -cardiac actin antisense (A) or sense (C); TEFR1 antisense (B) or sense (D). All dark-field photographs were taken at the same exposure level. Bar, 100 μ m.

(GST-TEFR1(1–38)), immunoprecipitation of both TEFR1b (lane 4) and mTEF-1 (lane 10) was blocked. In the presence of full-length mTEF-1 (GST-mTEF(1–430)), TEFR1b was immunoprecipitated (lane 5), while immunoprecipitation of mTEF-1 was blocked (lane 11). In the presence of GST-mTEF(1–29), neither immunoprecipitation of TEFR1b (lane 6) nor mTEF-1 (lane 12) was blocked. Therefore, although the polyclonal antibody showed cross-reactivity to mTEF-1, it contains activity

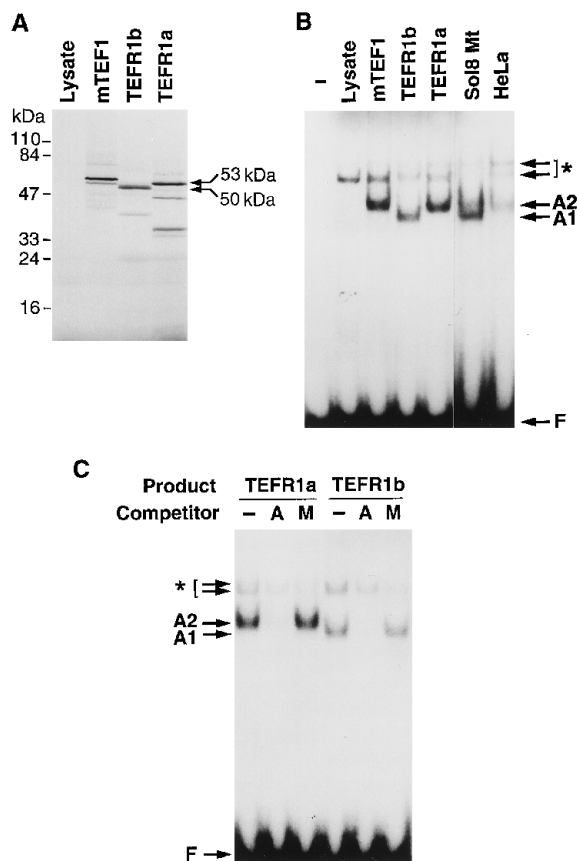


FIG. 6. *In vitro* transcription/translation of TEFR1 and mTEF-1. **A**, SDS-PAGE of *in vitro* translated mTEF-1 and TEFR1. The mTEF-1 and TEFR1a and 1b RNAs were synthesized *in vitro*, using either T7 or T3 RNA polymerase, and then translated using rabbit reticulocyte lysate. Three microliters of the *in vitro* translation products were separated on an 11% SDS-PAGE gel. **B**, gel mobility shift assay of *in vitro* translated products using end-labeled oligo A. Three microliters of either unprogrammed rabbit reticulocyte lysate (*Lysate*), mTEF-1, or TEFR1 translation products, or 2 μ g of nuclear extracts from HeLa cells or Sol8 myotubes (*Mt*) were incubated with end-labeled oligo A and separated on an 8% native PAGE gel. Arrows indicate the positions of specific complexes (A1 and A2), nonspecific complexes (*), and free probe (F). **C**, competition for complex formation between *in vitro* translated products and oligo A. TEFR1 translation products were incubated with end-labeled oligo A in the absence (–) or presence of unlabeled competitor oligo A or MutA (M) at a 50-fold molar excess over the labeled probe.

specific for TEFR1 (compare lanes 5 and 11).

We then conducted gel mobility shift assays using nuclear extracts from either HeLa cells or Sol8 myotubes in the presence of either preimmune or anti-TEFR1 antibody (Fig. 7C). Using Sol8 myotube nuclear extract, addition of anti-TEFR1 antibody resulted in the disappearance of the A1 complex band, reduction of the intensity of the A2 complex band, and formation of a supershifted complex (lanes 6 and 8). However, using HeLa cell nuclear extract, addition of anti-TEFR1 antibody reduced the formation of the A2 complex and did not result in the formation of a supershifted complex (lanes 2 and 4). Supershifted complexes are generally thought to contain probe, DNA-binding protein, and specific antibody. To confirm whether the muscle-specific A1 complex contains TEFR1, we examined the ability of GST-fusion protein competitors (Fig. 7A) to block the activity of the anti-TEFR1 antibody (Fig. 7D). A GST-fusion protein containing only TEFR1-specific residues (GST-TEFR1(1–24)) completely blocked the activity of the anti-TEFR1 antibody (lane 4), preventing the formation of the supershifted complex and returning the A1 and A2 complex band intensities to those observed in the presence of preimmune antibody (lane

1). The GST-TEFR1 immunogen (GST-TEFR1(1–38)) also completely blocked the activity of the anti-TEFR1 antibody (lane 3). In the presence of either GST alone (lane 6) or a GST-mTEF-1 competitor (GST-mTEF(1–29); lane 5), no competition was observed with the anti-TEFR1 antibody. These results suggest that the A1 complex contains TEFR1.

TEFR1 Contains an Activation Domain—We conducted transfection experiments in which TEFR1 expression vectors (pCMV-TEFR1a and 1b) were co-transfected with TK-CAT reporter constructs containing either GT-IIC or M-CAT elements upstream of the thymidine kinase promoter (Fig. 8). The activity of the CAT reporter genes in the absence of co-transfected TEFR1 is presumably due to endogenous M-CAT-binding factors (mTEF-1 and/or TEFR1). When TEFR1 was co-transfected, this background CAT activity was repressed in a dose-dependent manner in both HeLa cells and Sol8 myotubes. Based on the close structural similarity and sequence identity between TEFR1 and mTEF-1, this phenomenon might be due to “squenching” (see “Discussion”). Because the M-CAT motif has been shown to be a positive-regulatory element in multiple muscle-specific genes (6, 7, 9), we conducted experiments to determine whether TEFR1 contains an activation domain. We conducted transfection experiments using GAL4/TEFR1 chimeras and a GAL4-CAT reporter, an approach which has been used successfully in the study of TEF-1 activation function (26). The full coding regions of TEFR1a and 1b were connected in-frame to the DNA-binding domain of yeast activator GAL4 (aa 1–147) to form GAL4-TEFR 1 and GAL4-TEFR 2, respectively (Fig. 9). Co-transfection of GAL4-TEFR 1 with the p3xGAL4-BGCAT reporter (kindly provided by Dr. Erik K. Flemington) slightly increased basal CAT activity in Sol8 myotubes (5-fold) and HeLa cells (2-fold). GAL4-TEFR 2 showed more activation than GAL4-TEFR 1 in Sol8 myotubes (44-fold) and HeLa cells (6-fold).

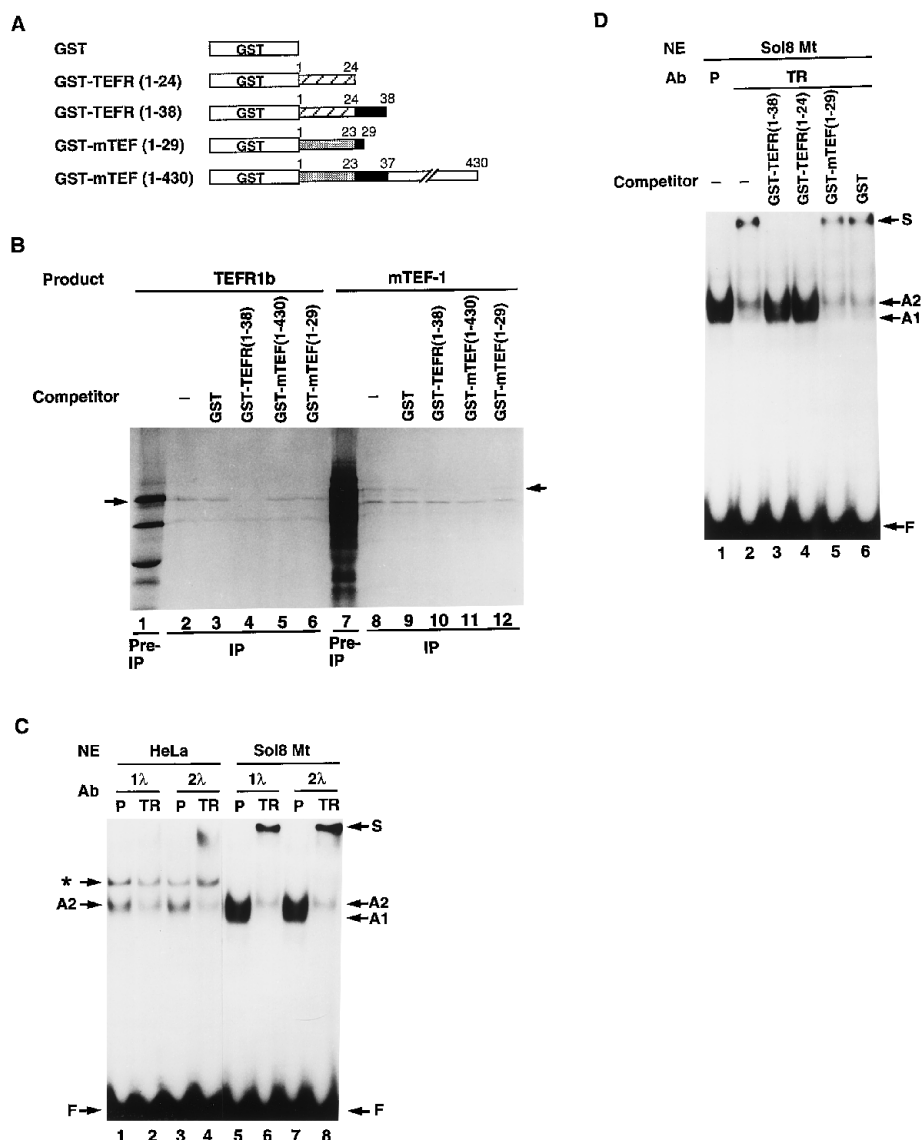
There are two functional DNA-binding domains in both GAL4-TEFR 1 and GAL4-TEFR 2. It has been reported that GAL4/AP2, SP1/(CTF/NF1), and GAL4/TEF-1 chimeras, which contain more than one functional DNA-binding domain, are weak transactivators, although they contain strong activation domains (26, 41, 42). To determine whether or not strong activation functions in GAL4-TEFR 1 and 2 were being masked by the presence of two functional DNA-binding domains, we mutated two conserved amino acids within the first putative α -helix of the TEFR1 TEA DNA-binding domain, aa 48 (Leu \rightarrow Pro) and 50 (Ile \rightarrow Phe) (Fig. 1D). Mutations in this region result in loss of DNA-binding activity (26) (data not shown). Transfection of GAL4-TEFR 1M or GAL4-TEFR 2M which contain mutated TEFR1a and 1b, respectively, strongly activated the expression of the p3xGAL4-BGCAT reporter in both Sol8 myotubes (\sim 100-fold) and HeLa cells (\sim 150-fold) (Fig. 9). Deletion of aa 1–111 of TEFR1a (GAL4-TEFR 3), including acidic and basic regions and the entire TEA domain, dramatically increased the expression of the reporter plasmid (\sim 400 fold in Sol8; \sim 800-fold in HeLa). Strong activation was still observed when aa 1–206, including the proline-rich region, were deleted (GAL4-TEFR 5). However, deletion of aa 1–302, including one of two STY-rich regions, resulted in total loss of activation function (GAL4-TEFR 6). In addition, there was no activation when the C-terminal 28 aa (aa 400–427) were removed from GAL4-TEFR 4 (GAL4-TEFR 7). Therefore, TEFR1 apparently contains one or more activation domains, at least one of which is located in the C-terminal half of the protein (aa 207–427).

DISCUSSION

TEF-1 is considered to be the transcription factor that is primarily responsible for the transcriptional activation

FIG. 7. Relationship between TEFR1 and M-CAT-binding factors.

A, structure of GST-fusion proteins. Numbers indicate positions of amino acids in either TEFR1 or mTEF-1. Regions of identity between TEFR1 and mTEF-1 are indicated by solid bars. Hatched and shaded bars indicate specific regions of TEFR1 and mTEF-1, respectively. **B**, immunoprecipitation (IP) of TEFR1 and mTEF1 by anti-TEFR1 antibody. The *in vitro* transcription/translation products from TEFR1b and mTEF-1 cDNA clones (Pre-IP) were immunoprecipitated by anti-TEFR1 antibody in the absence (–) or presence of an equimolar amount of GST-fusion protein competitors shown in **A**. Proteins were separated on an 11% SDS-PAGE gel. Arrows indicate bands corresponding to full-length of TEFR1b and mTEF-1. **C** and **D**, effect of anti-TEFR1 antibody (Ab) on the binding of nuclear factors to oligo A (M-CAT motif). Three micrograms of nuclear extract (NE) from either HeLa cells or Sol8 myotubes (Mt) were preincubated with preimmune (P) or anti-TEFR1 (TR) IgY in the absence (all of C; – in D) or presence of GST-fusion protein competitors shown in **A**. Either 1 or 2 µl of 6 mg/ml IgY were used. Reaction mixtures were further incubated with end-labeled oligo A and separated on an 8% native PAGE gel. Arrows indicate the position of specific complexes (A1 and A2), nonspecific complex (*), supershifted complexes (S), and free probe (F).



through the M-CAT cis-regulatory element found in certain muscle-specific genes. This seems reasonable for both non-muscle and cardiomyocyte cells, as in both cases there is a single M-CAT-binding activity seen in gel mobility shift assays, which has been attributed to TEF-1 by antigenic criteria (7, 13, 17, 19). However, we had shown previously that in differentiated mouse skeletal muscle in culture two M-CAT-binding activities could be detected, which we designated A1 and A2 (29). The M-CAT-A2 factor-complex is ubiquitous and contains mTEF-1 as a major component (20). Here we report the isolation of a TEF-1-related transcription factor, TEFR1, which appears to be a major component of the muscle-specific M-CAT-A1 factor-complex.

We have isolated two TEFR1 cDNAs, TEFR1a and 1b, which have identical nucleotide sequences within their coding and proximal noncoding regions, but for the absence of 129 nt (43 aa) in TEFR1b. Southern blotting of CD1-mouse genomic DNA with the full-length TEFR1b cDNA clone showed hybridization to a single, ~10-kb *EcoRI* fragment, which suggests that TEFR1a and 1b are splice forms of a single gene (data not shown). In all cell types and tissues examined by RT-PCR, TEFR1b transcripts have been more abundant than 1a, except in the case of 3T3 cells, where the ratio is inverted (Fig. 3). It has been reported that the function of a transcription factor can

be altered by structural changes occurring *in vivo*, such as alternative splicing (43, 44) and phosphorylation (45). Further work will be required to determine whether TEFR1a and 1b are functionally distinct.

TEFR1 appears to use AUU (Ile) at nt 110 as an initiation codon, similar to TEF-1 (17). Mutation of this codon to UGG (Trp) resulted in no translational initiation *in vitro* from nt 110, while mutation to AUG (Met) increased translational efficiency (data not shown). There are several AUG codons in the 5' portion of the TEFR1 cDNAs. However, comparison of the flanking sequences surrounding these various codons indicated that the AUU at nt 110 lies in the best Kozak sequence context (46). Furthermore, none of the AUG codons is appropriately positioned to account for the fact that TEFR1 contains a TEA DNA-binding domain, as indicated by its ability to bind to the M-CAT motif (Fig. 6). Also, only initiation at the AUU codon at nt 110 can account for both the molecular weights of the *in vitro* translated TEFR1 products (Fig. 6) and the existence of the amino-terminal residues which were detected by the anti-TEFR1 antibody (Fig. 7).

TEFR1 is the third member of the TEA family to be found in mammals. The expression patterns of ETF (23) and TEFR1 are tissue-restricted, in contrast to the widespread expression of mTEF-1 (20). Comparison of the predicted amino acid se-

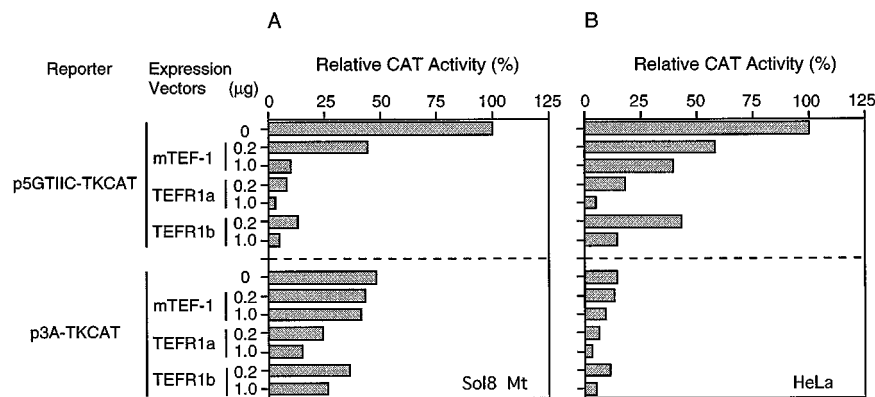


FIG. 8. **Effect of overexpression of TEFR1 and mTEF-1 on reporter plasmids containing multiple binding sites.** Each reporter (2 µg) was co-transfected with expression vector (0.2 or 1.0 µg) and pCMV-lacZ (1 µg) into either Sol8 myocytes (A) or HeLa cells (B). The 5GTIIC-TKCAT and 3A-TKCAT plasmids contain five copies of a GTIIC tandem repeat and three copies of the A element (21 bp), respectively, upstream of the herpes simplex virus thymidine kinase promoter in pTKCAT (20). The CAT activity was normalized using the β-galactosidase activity for each transfection. All CAT activities are given relative to the value obtained for the p5GTIIC-TKCAT, which is set at 100% for each cell type. Results represent the average of two to four separate transfection experiments. Variation of normalized values was less than 10% between experiments.

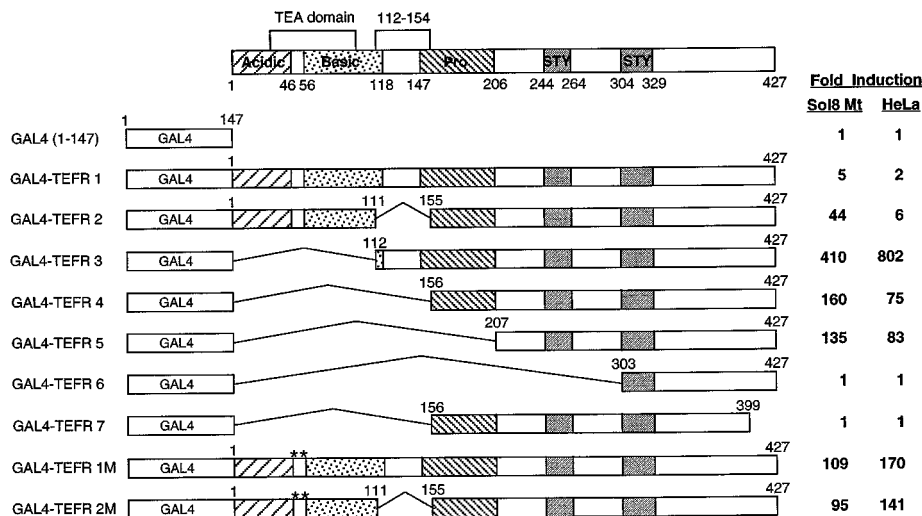


FIG. 9. **Transactivation of the p3xGAL4-BGCAT reporter by GAL4/TEFR1-chimeric proteins.** The p3xGAL4-BGCAT reporter (2 µg) containing three copies of the GAL4 DNA-binding site was transfected with each expression vector for a GAL4/TEFR1 chimera (0.1 µg) and pCMV-lacZ (1 µg) into either Sol8 myocytes or HeLa cells. CAT activity was normalized using the β-galactosidase activity for each transfection. Values for fold induction are given relative to activity of the GAL4(1-147) construct. Results represent the average of two to four separate transfection experiments. Variation of normalized values was less than 10% between experiments. The structure of TEFR1a is shown at the top. The region absent from TEFR1b (aa 112-154) and the TEA domain (aa 31-98) are shown above the TEFR1a diagram. The acidic, basic, proline-rich (Pro), and serine-threonine-tyrosine-rich (STY) regions are indicated by different fill patterns. The positions of mutated aa 48 (Leu → Pro) and aa 50 (Ile → Phe) in GAL4-TEFR 1M and 2M are indicated by asterisks.

quences of mTEF-1, TEFR1, and ETF indicated that ETF is equally divergent, ~65% identity, from either mTEF-1 or TEFR1, while mTEF-1 and TEFR1 share 76% identity. Comparison of TEFR1 to the 172-bp EST 683 human cDNA clone 32B5 (GenBank™ accession no. T25108) (47) showed that they share 95% identity at the amino acid level. The sequence of EST 683, which was identified by systematic sequencing of a colorectal cancer cDNA library, coincides with TEFR1 aa 261-316. EST 683 might correspond to the human analog of mouse TEFR1, but this must be confirmed by cloning and sequencing of the full-length cDNA. EST 683 also shows a high degree of homology to chicken TEF-1 (cTEF-1) (21). Comparison of the cTEF-1 predicted amino acid and nucleotide sequences to those of mTEF-1 and TEFR1 revealed an unexpected result. At the amino acid level, TEFR1 has an overall identity with cTEF-1 of 87%, but only 76% with mTEF-1, while mTEF-1 and cTEF-1 share 77% identity. Comparison of the nucleotide sequences of the TEA domains revealed that TEFR1 shares 83% identity with cTEF-1, but only 76% with mTEF-1, while mTEF-1 and

cTEF-1 share 78% identity. These comparisons suggest that cTEF-1 is the chicken analog of TEFR1 rather than mTEF-1. However, the structures of the activation domains of TEFR1 (Fig. 9) and cTEF-1 (21) are significantly different. One possibility is that cTEF-1, mTEF-1, and TEFR1 represent three distinct members of the TEA family. If, on the other hand, cTEF-1 and TEFR1 are homologs, then differences between the activation domains of cTEF-1 and TEFR1 might be due to differences in co-factors between avians and mammals (see below). A third alternative is that, in avians, there might be a single transcription factor (cTEF-1) responsible for transcriptional activation through the M-CAT motif in all striated muscle. TEFR1 contains multiple putative activation domains, most of which are shared structurally with TEF-1, such as acidic (48, 49), proline-rich (41, 42), and STY-rich (50, 51) regions. However, the acidic and proline-rich regions which are essential for TEF-1 activation function (26) are dispensable for TEFR1 activation function (Fig. 9). In transfection assays using TEFR1

expression vectors and CAT reporter constructs containing either M-CAT or GT-IIC elements, we observed a dose-dependent repression of reporter gene activity (Fig. 8). One possible explanation for this observation is that TEFR1 is an M-CAT-binding repressor. However, two observations make this possibility unlikely. First, accumulation of transcripts of some contractile proteins in differentiated skeletal muscle is dependent upon the M-CAT positive cis-regulatory element. Second, the formation of the M-CAT-A1 factor (TEFR1) complex occurs in skeletal muscle *in vitro* as differentiation proceeds (29). An alternative explanation is that overexpression of TEFR1 results in "squenching" (52), which implies that TEFR1 requires a co-activator(s) to function, as has been determined biochemically for TEF-1 (27, 28). The dose-dependence of the squenching effect and minimal structural requirements for activation are different between TEF-1 and TEFR1. Therefore, TEF-1 and TEFR1 might interact with overlapping but nonidentical sets of co-factors. Further work will be required to determine whether the structural differences between TEF-1 and TEFR1 indicate *in vivo* functional differences.

To directly address the question of whether TEFR1 is a component of the muscle-specific A1 complex, we generated a polyclonal antibody to the amino terminus of TEFR1. Gel mobility shift assays in the presence of this antibody resulted in the formation of a supershifted complex and reduction in both A1 and A2 complex band intensities (Fig. 7C). This effect could be eliminated by competition with a segment of the immunogen which was specific to TEFR1 (GST-TEFR(1–24); Fig. 7D, lane 4). These results, along with previous data indicating that components of the A1 complex are antigenically distinct from TEF-1 (20), suggest that a major component of the muscle-specific A1 complex is TEFR1. It is possible that the A1 complex does not contain TEFR1, but rather a protein that contains a region that is antigenically identical to the amino terminus of TEFR1. This is unlikely, though, because the region corresponding to TEFR(1–24) is highly divergent among the three mammalian TEA-domain proteins identified so far, TEF-1 (17, 20, 22), TEFR1, and ETF (23). Further work will be required to determine whether the A2 complex in differentiated skeletal muscle also contains TEFR1 and to determine whether TEFR1a, 1b, or both are present in the muscle-specific A1 complex.

Skeletal muscle cell lines exhibit many of the properties of embryonic and perinatal muscle (53, 54). The enrichment of TEFR1 transcripts in differentiated skeletal muscle cells in culture suggested that TEFR1 might also be enriched in embryonic skeletal muscle. *In situ* hybridization analysis of mouse embryos at embryonic day 9 (Fig. 4) and embryonic day 14.5 (Fig. 5) showed that TEFR1 transcripts are relatively abundant in the skeletal myogenic lineage. Our Northern analysis of adult tissue RNA showed that TEFR1 transcripts are expressed weakly in adult skeletal muscle. Therefore, TEFR1 might be transiently required for some early stage of myofiber maturation, such as appropriate accumulation of key components of the contractile apparatus.

Northern analysis showed that both mTEF-1 (20) and TEFR1 (Fig. 2B) are present in adult heart. Our *in situ* hybridization results showed no enrichment of TEFR1 transcripts in embryonic heart (data not shown). Our initial isolation of TEFR1 was from an adult mouse cardiac cDNA library. However, after multiple screenings of this library, only a single, partial TEFR1 cDNA clone was isolated, while we recovered multiple mTEF-1 cDNA clones. This suggests that TEFR1 transcripts are less abundant than those of mTEF-1 in the adult heart. Also, there appears to be a single cardiac M-CAT-binding activity (7, 13, 19). Although these observations imply

a minor role for TEFR1 in the heart, we cannot rule out the possibility that TEFR1 is involved in cardiac gene regulation. In particular, we do not know whether TEFR1 transcripts are present at the time when contractile protein gene transcription is initiated in the heart, prior to embryonic day 8 (34).

In the adult mouse, TEFR1 transcripts are present at a higher level in lung than in any other tissue we tested. Mouse TEF-1 transcripts are also present at a high level in adult lung, approximately equivalent to striated muscle and kidney (20). Our *in situ* hybridization data indicated that TEFR1 is not enriched in the embryonic lung at either embryonic day 9 or 14.5 (data not shown). This suggests that TEFR1 might not be involved in early development of the lung. Though TEFR1 and possibly other members of the TEA family might be involved in lung-specific gene expression, especially postnatally, no lung-specific promoters have been identified yet that contain functional M-CAT motifs.

As more transcription factors have been characterized, it has become clear that the existence of networks of factors interacting at single cis-regulatory elements is common. The E-box and A/T-rich motifs have been shown to interact with distinct families of transcription factors, the MyoD/bHLH (1) and MEF-2/MADS (2) families, respectively. The M-CAT motif appears to be another such element, interacting with members of the TEA family of transcription factors. Our results indicate that TEFR1 and TEF-1 are closely related members of this TEA family and that TEFR1 might play a role in the embryonic development of skeletal muscle.

Acknowledgments—We thank Dr. Erik K. Flemington (Dana Farber Cancer Institute, Boston, MA) for providing plasmids BS-(RSV)-GAL4(1–147)-E2F(Δ1–367) and 3xGAL4-BGCAT. We also thank Drs. Pierre Chambon and Irwin Davidson (Institut de Chimie Biologique, Strasbourg, France) for providing human TEF-1 cDNA.

REFERENCES

- Weintraub, H. (1993) *Cell* **75**, 1241–1244
- Yu, Y.-T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) *Genes & Dev.* **6**, 1783–1798
- Olson, E. (1993) *Circ. Res.* **72**, 1–6
- Chambers, A. E., Logan, M., Kotecha, S., Towers, N., Sparrow, D., and Mohun, T. J. (1994) *Genes & Dev.* **8**, 1324–1334
- Lilly, B., Galesky, S., Firulli, A. B., Schulz, R. A., and Olson, E. N. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5662–5666
- Mar, J. H., and Ordahl, C. P. (1990) *Mol. Cell. Biol.* **10**, 4271–4283
- MacLellan, W. R., Lee, T.-C., Schwartz, R. J., and Schneider, M. D. (1994) *J. Biol. Chem.* **269**, 16754–16760
- Sartorelli, V., Webster, L. A., and Kedes, L. (1990) *Genes & Dev.* **4**, 1811–1822
- Shimizu, N., Prior, G., Umeda, P. K., and Zak, R. (1992) *Nucleic Acids Res.* **20**, 1793–1799
- Thompson, W. R., Nadal-Ginard, B., and Mahdavi, V. (1991) *J. Biol. Chem.* **266**, 22678–22688
- Flink, I. L., Edwards, J. G., Bahl, J. J., Liew, C.-C., Sole, M., and Morkin, E. (1992) *J. Biol. Chem.* **267**, 9917–9924
- Knotts, S., Rindt, H., Neumann, J., and Robbins, J. (1994) *J. Biol. Chem.* **269**, 31275–31282
- Molkentin, J. D., and Markham, B. E. (1994) *Mol. Cell. Biol.* **14**, 5056–5065
- Davidson, I., Xiao, J. H., Rosales, R., Staub, A., and Chambon, P. (1988) *Cell* **54**, 931–942
- Ishiji, T., Lace, M. J., Parkkinen, S., Anderson, R. D., Haugen, T. H., Cripe, T. P., Xiao, J.-H., Davidson, I., Chambon, P., and Turek, L. P. (1992) *EMBO J.* **6**, 2271–2281
- Mélin, F., Miranda, M., Montreau, N., DePamphilis, M. L., and Blangy, D. (1993) *EMBO J.* **12**, 4657–4666
- Xiao, J. H., Davidson, I., Matthes, H., Garnier, J.-M., and Chambon, P. (1991) *Cell* **65**, 551–568
- Farrance, I. K. G., Mar, J. H., and Ordahl, C. P. (1992) *J. Biol. Chem.* **267**, 17234–17240
- Kariya, K., Farrance, I. K. G., and Simpson, P. C. (1993) *J. Biol. Chem.* **268**, 26658–26662
- Shimizu, N., Smith, G., and Izumo, S. (1993) *Nucleic Acids Res.* **21**, 4103–4110
- Stewart, A. F. R., Larkin, S. B., Farrance, I. K. G., Mar, J. H., Hall, D. E., and Ordahl, C. P. (1994) *J. Biol. Chem.* **269**, 3147–3150
- Blatt, C., and DePamphilis, M. L. (1993) *Nucleic Acids Res.* **21**, 747–748
- Yasunami, M., Suzuki, H., Houtani, T., Sugimoto, T., and Ohkubo, H. (1995) *J. Biol. Chem.* **270**, 18649–18654
- Chen, Z., Friedrich, G. A., and Soriano, P. (1994) *Genes & Dev.* **8**, 2293–2301
- Bürglin, T. R. (1991) *Cell* **66**, 11–12
- Hwang, J.-J., Chambon, P., and Davidson, I. (1993) *EMBO J.* **12**, 2337–2348
- Brou, C., Chaudhary, S., Davidson, I., Lutz, Y., Wu, J., Egly, J.-M., Tora, J., and Chambon, P. (1993) *EMBO J.* **12**, 489–499

28. Chaudhary, S., Tora, L., and Davidson, I. (1995) *J. Biol. Chem.* **270**, 3631–3637
29. Shimizu, N., Dizon, E., and Zak, R. (1992) *Mol. Cell. Biol.* **12**, 619–630
30. Genetics Computer Group, Inc. (1991) *Program Manual for the GCG Package*, Version 7, Genetics Computer Group, Inc., Madison, WI
31. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
32. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
33. Parnes, J. R., and Seidman, J. G. (1982) *Cell* **29**, 661–669
34. Sassoon, D. A., Garner, I., and Buckingham, M. (1988) *Development* **104**, 155–164
35. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York
36. Cox, K. H., DeLeon, D. V., Angerer, L. M., and Angerer, R. C. (1984) *Dev. Biol.* **101**, 485–502
37. Laemmli, U. K. (1970) *Nature* **227**, 680–685
38. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 1373–1376
39. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
40. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
41. Mermod, N., O'Neill, E. A., Kelly, T. J., and Tjian, R. (1989) *Cell* **58**, 741–753
42. Williams, T., and Tjian, R. (1991) *Genes & Dev.* **5**, 670–682
43. Foulkes, N. S., and Sassone-Corsi, P. (1992) *Cell* **68**, 411–414
44. Georgopoulos, K., Morgan, B. A., and Moore, D. D. (1992) *Mol. Cell. Biol.* **12**, 747–757
45. Hunter, T., and Karin, M. (1992) *Cell* **70**, 375–387
46. Kozak, M. (1991) *J. Cell Biol.* **115**, 887–903
47. Frigerio, J. M., Berthéze, P., Garrido, P., Ortiz, E., Barthelémy, S., Vasseur, S., Sastre, B., Seleznieff, I., Dagorn, J.-C., and Iovanna, J. L. (1995) *Hum. Mol. Genet.* **4**, 37–43
48. Hope, I. A., Mahadevan, S., and Struhl, K. (1988) *Nature* **333**, 635–640
49. Ma, J., and Ptashne, M. (1987) *Cell* **51**, 113–119
50. Seipel, K., Georgiev, O., and Schaffner, W. (1992) *EMBO J.* **11**, 4961–4968
51. Theill, L. E., Castrillo, J.-L., Wu, D., and Karin, M. (1989) *Nature* **342**, 945–948
52. Clark, A. R., and Docherty, K. (1993) *Biochem. J.* **296**, 521–541
53. Silberstein, L., Webster, S. G., Travis, M., and Blau, H. M. (1986) *Cell* **46**, 1075–1081
54. Weydert, A., Barton, P., Harris, A. J., Pinset, C., and Buckingham, M. (1987) *Cell* **49**, 121–129
55. Campbell, S., Inamdar, M., Rodrigues, V., Raghavan, V., Palazzolo, M., and Chovnick, A. (1992) *Genes & Dev.* **6**, 367–379
56. Laloux, I., Dubois, E., Dewerchin, M., and Jacobs, E. (1990) *Mol. Cell. Biol.* **10**, 3541–3550
57. Mirabito, P. M., Adams, T. H., and Timberlake, W. E. (1989) *Cell* **57**, 859–868