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A Novel Family of Developmentally Regulated Mammalian Transcription Factors Containing the TEA/ATTS DNA Binding Domain*

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We describe the molecular cloning of two novel human and murine transcription factors containing the TEA/ATTS DNA binding domain and related to transcriptional enhancer factor-1 (TEF-1). These factors bind to the consensus TEA/ATTS cognate binding site exemplified by the GT-IIC and Sph enhansons of the SV40 enhancer but differ in their ability to bind cooperatively to tandemly repeated sites. The human TEFs are differentially expressed in cultured cell lines and the mouse (m)TEFs are differentially expressed in embryonic and extra-embryonic tissues in early post-implantation embryos. Strikingly, at later stages of embryogenesis, mTEF-3 is specifically expressed in skeletal muscle precursors, whereas mTEF-1 is expressed not only in developing skeletal muscle but also in the myocardium. Together with previous data, these results point to important, partially redundant, roles for these TEF proteins in myogenesis and cardiogenesis. In addition, mTEF-1 is strongly coexpressed with mTEF-4 in mitotic neuroblasts, while accentuated mTEF-4 expression is also observed in the gut and the nephrogenic region of the kidney. These observations suggest additional roles for the TEF proteins in central nervous system development and organogenesis.

Transcriptional enhancer factor-1 (TEF-1)[1] belongs to a family of proteins sharing the TEA/ATTS domain that shows a remarkable degree of conservation between yeast and humans (Refs. 1–4 and see Fig. 1). In addition to TEF-1, this family comprises the yeast protein TEC1 that is postulated to bind to the sterile responsive element downstream from the Ty1 transposon long terminal repeat (5, 6), and the *Aspergillus nidulans* factor *Aba*A, controlling a regulatory circuit in the terminal stage of conidiophore development essential for spore formation (7, 8 and Refs. therein). In *Drosophila*, the TEF-1 homologue *scalloped* (*sd*) is expressed in the regions of the wing disc that are destined to become defective structures in viable sd mutants (9). *Sd* is also expressed in the embryonic central nervous system (CNS) and in peripheral sense organs. In the adult brain expression is restricted to subsets of cells some of which are involved in taste responses. Accordingly, viable *sd* mutants also display abnormal taste behavior (10). In mouse, a TEF-1-related factor, ETF, has been recently described (11). Like *sd*, ETF was reported to be specifically expressed in the developing brain.

The most studied member of the TEA/ATTS family, TEF-1, is a transcriptional activator first identified in HeLa cells by its binding to the GT-IIC and Sph(I + II) enhansons in the simian virus 40 (SV40) enhancer (1, 12). We have shown that the TEF-1 TEA/ATTS domain is the minimal domain required for specific binding to the GT-IIC and Sph enhansons (13). However, in both TEF-1 and *sd*, DNA binding is modulated by sequences outside of the TEA/ATTS DNA binding domain (DBD) (13). Comparison of TEF-1 binding sites from a variety of enhancers (see below) shows that it binds to highly degenerate sequences (consensus 5′-(A/T)(A/G)(A/G)(A/T)ATG(C/T)N-3′). A similar consensus sequence has been deduced from the comparison of *Aba*A binding sites (8). In contrast to the majority of sequence-specific DNA binding proteins that bind cooperatively to palindromic elements, TEF-1 binds cooperatively to tandem, but not spaced or inverted, repeats of its binding sites (1). Consequently, tandemly repeated TEF-1 binding sites have higher enhancer activity *in vivo* than spaced repeats (14, 15).

TEF-1 not only activates transcription from the SV40 early promoter, but together with large T antigen it acts to modulate SV40 late transcription (16–20). Transcription of the human papilloma virus 16 E6/E7 oncogenes from the P97 promoter is also in part regulated by TEF-1 (21). Transcriptional activation by TEF-1 requires the cooperative action of several regions of the protein (13), a limiting transcriptional intermediary factor(s) (2, 21), and, *in vitro*, TATA-binding protein (TBP)-associated factors ($hTAF_{II}s$) present in two chromatographically distinct TFIID complexes (22). TEF-1 activity is also subject to negative regulation by at least two distinct negatively acting factors (23, 24).

TEF-1 binding sites have also been characterized in several

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X94438, X94441, X94440, X94442.

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[1] The abbreviations used are: TEF-1, transcriptional enhancer factor-1; dpc, days post-coitum; RT, reverse transcription; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assays; CNS, central nervous system; ORF, open reading frame; DBD, DNA binding domain; h, human; m, murine.

muscle-specific promoters. Expression of the cardiac troponin C and myosin α and β heavy chain genes has been shown to depend on a GT-IIC-related enhanson (M-CAT) (25–31). This has been demonstrated not only in gene transfer experiments but also, in the case of the β-myosin heavy chain gene, in transgenic mice (31). The M-CAT enhanson is recognized by M-CAT binding factor, subsequently shown to be antigenically related to TEF-1 (26). A putative chicken homologue of TEF-1 has been isolated and shown to bind to the M-CAT enhanson (cTEF-1, 32). Furthermore, muscle-enriched isoforms of cTEF, which have altered transactivation properties, have been identified. TEF-1 binding sites have also been shown to regulate the expression of vascular smooth muscle α-actin (33) and are involved in the reactivation of the β isoform of myosin heavy chain and skeletal α-actin genes upon induced cardiac hypertrophy (34–37). The latter genes are normally expressed during fetal cardiac development suggesting that TEF-1 contributes to the expression of these genes during cardiogenesis. Indeed mice in which the murine homologue of TEF-1 has been disrupted die during embryogenesis due to heart malformation, further supporting the idea that TEF-1 is of critical importance for cardiac development (38). Aside from muscle-specific gene regulation, putative TEF-1 binding sites have been noted in the placental-specific human chorionic somatomammotropin (hCS also called placental lactogen) -B gene enhancer (39–44).

Here we describe two novel human and murine factors belonging to the TEA/ATTS family and related to TEF-1. These proteins bind to the GT-IIC and Sph enhansons of SV40 but differ in their ability to bind cooperatively to tandemly repeated sites. However, cooperative binding to tandem sites is an intrinsic property of the minimal TEA/ATTS DBD showing that differences elsewhere in the TEF factors modulate their DNA binding properties. Reverse transcription PCR experiments show differential expression of the human TEF factors in cultured cell lines. *In situ* hybridization shows distinct expression patterns of the mTEFs in embryonic and extra-embryonic tissues prior to gastrulation. From mid-gestational stages onward strong mTEF-1 expression is observed in developing skeletal muscle and myocardium, but mTEF-3 is expressed almost exclusively in skeletal muscle precursors. These results, together with those of previous studies, highlight the important roles played by the TEF proteins in myogenesis and cardiogenesis. However, mTEF-1 expression is not limited to skeletal and heart muscle as it is strongly expressed along with mTEF-4 in mitotic neuroblasts both in the brain and spinal cord. At later stages of embryogenesis mTEF-1 and mTEF-4 show distinct expression patterns in several developing structures such as the olfactory system, the intestine, and the kidney.

MATERIALS AND METHODS

Polymerase Chain Reaction Amplification and Screening of cDNA Libraries—Two degenerate oligonucleotides (5′-CCCAAGCTTGGC(A/C)G GAA(C/T)GA(A/G)(C/T)TGAT(A/C)GC-3′ and 5′-CCCAAGCTTC(A/G/C/T)A(G/A)(A/G/C/T)AC(C/T)TG(T/G/A)AT(G/A)TG-3′) corresponding to the TEA domain amino acid sequences GRNELIA and HIQVL were used to PCR-amplify a series of cDNA libraries including those from HeLa cells, human fetal brain, retinoic acid-differentiated mouse embryonic stem cells, and 10.5 dpc mouse embryos. 30 cycles (1 min at 94 °C, 1.5 min at 40 °C, and 1.5 min at 72 °C) of PCR were performed under standard conditions in a 100-μl reaction volume with 200 pmol of each degenerate oligonucleotide primer, 2–4-μl aliquots of each library ($>10^9$ plaque forming units/ml) and 2 units of ampliTaq polymerase (Perkin Elmer). Amplification products of the correct size were gel-purified and cloned into the TA cloning vector (Invitrogen). DNA sequencing was performed on an Applied Biosystems automated sequencer. TEF-specific probes for screening cDNA libraries were generated by PCR using the degenerate primers and the partial cDNAs as templates in the presence of [α-^{32}P]dCTP. cDNA libraries were screened by hybridization at 42 °C in 6 × SSC, 50% formamide. Filters were washed at 55 °C in 3 × SSC. Positive clones were picked, purified, and excised from λZAPII (Stratagene) libraries by standard procedures. The DNA sequences of both strands of each clone were determined using internal primers. DNA and protein sequence analysis were performed using the GCG (Genetics Computer Group, University of Wisconsin) software package.

Construction of Expression Vectors—The ORFs for the novel TEFs were amplified with appropriately positioned oligonucleotides containing a consensus Kozak sequence replacing the translation initiation isoleucine codons with ATG. The primers contained *Eco*RI/*Xba*I or *Eco*RI/*Xho*I restriction sites, and the PCR fragments were cloned between the corresponding sites in pXJ41. The DNA sequences of the expression vectors were verified by automated DNA sequencing.

Transfections and Preparation of Cell Extracts—Cos cells were transfected by the calcium phosphate coprecipitation technique as described previously (13). 48 h after transfection the cells were harvested (from 60-mm diameter dishes) and extracts prepared by three cycles of freeze-thaw in 200 μl of buffer A (50 mM Tris-HCl, pH 7.9, 20% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, and 1 mM dithiothreitol) containing 0.5 M KCl and 2.5 μg/ml leupeptin, pepstatin, aprotinin, antipain, and chymostatin as described (45, 46). Generally between 2 and 8 μl of the extracts were then used in EMSA.

Cloning and Expression of the Histidine-tagged TEF-1 TEA Domain—The region of TEF-1 encoding amino acids 28–104 was PCR-amplified with primers containing *Nde*I and *Bam*HI restriction sites. The PCR product was cloned between the *Nde*I and *Bam*HI sites of the pET15 vector to generate a TEA domain tagged with 6 histidines (6His-TEA). The plasmid was transformed into the BL21 strain, and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside for 1.5 h. From 400 ml of culture 20 ml of bacterial extract was prepared in buffer A containing 0.5 M KCl and protease inhibitors, and the fusion protein was purified by chromatography on a 2-ml nitrilotriacetic acid-Ni-agarose column (Qiagen). The column was washed sequentially with 4-column volumes of buffer A containing 1.0 M KCl and buffer A containing 0.05 M KCl and 0.05 M imidazole. The fusion protein was then eluted with 2 × 1.5 ml of buffer A containing 0.05 M KCl and 0.25 M imidazole and loaded onto a 1-ml double-stranded DNA cellulose column (Sigma) that was washed extensively with buffer A containing 0.25 M KCl. The protein was then eluted in 3 × 1 ml of buffer A containing 0.8 M KCl, dialyzed against buffer A containing 0.1 M KCl, and frozen in aliquots at −80 °C.

Electrophoretic Mobility Shift Assays—The oligonucleotides containing the wild-type or mutated GT-IIC enhanson and the tandemly repeated GT-IIC or Sph enhansons, or repeats spaced by 5 or 10 nucleotides were as described previously (1, 13). The oligonucleotides were ^{32}P-5′-end-labeled using polynucleotide kinase and separated from unincorporated [γ-^{32}P]ATP by chromatography of G-50-Sepharose. Electrophoretic mobility shift assays (EMSA) were performed essentially as described previously (13) on 6% polyacrylamide gels in 0.5 × standard TBE buffer.

Reverse Transcription PCR—Total cytoplasmic RNA was isolated from human cell lines by lysis with buffer B (50 mM Tris-HCl, pH 7.9, 0.1 M KCl, 0.5 mM EDTA, and 0.2% Nonidet P-40) and subsequent phenol/chloroform extractions and ethanol precipitations. To test the integrity of the RNA preparations RT-PCR was performed using primers in the hRBP17 subunit of the RNA polymerases generating a 630-nucleotide fragment (Ref. 47 and data not shown). Reverse transcription was performed with 2.5 μg of RNA for 30 min at 40 °C with 5 units of Moloney murine leukemia virus reverse transcriptase using the following TEF-specific antisense primers; hTEF-1, 5′-CTTTAGCTTGGAATGAAAA-3′; hTEF-3, 5′-AGCTTGGCCTGGATCTCG-3′; hTEF-4, 5′-CTTGGACTGGATTTCCCT-3. The products of reverse transcription were then amplified using the same antisense primers and the following sense primers: hTEF-1, 5′-GACTCTGCAGATAAGCCA-3′; hTEF-3, 5′-AGTGGAGCTCTCCCACCT-3′; hTEF-4, 5′-GGGGGTGACGGGGGCCCG-3′. The 5′ and 3′ primers were chosen in separate exons to distinguish the cDNA product from contaminating genomic DNA. RT-PCR generated a 270-nucleotide fragment for hTEF-1, 316 nucleotides for hTEF-3, and 255 nucleotides for hTEF-4. Control PCR reactions were performed using 10 pg of the appropriate expression vectors. 30 cycles of PCR were performed for 1 min at 94 °C, 1.5 min at 53 °C, and 1.5 min at 72 °C in a 60-μl volume. 15 μl of the reaction was then electrophoresed, transferred to nitrocellulose, and hybridized to the homologous ^{32}P-5′-end-labeled TEA domain probes generated by PCR using the degenerate oligonucleotide primers shown in Fig. 1.

In Situ Hybridization—The mTEF antisense riboprobes were generated by *in vitro* transcription of the Bluescript SK plasmids recovered

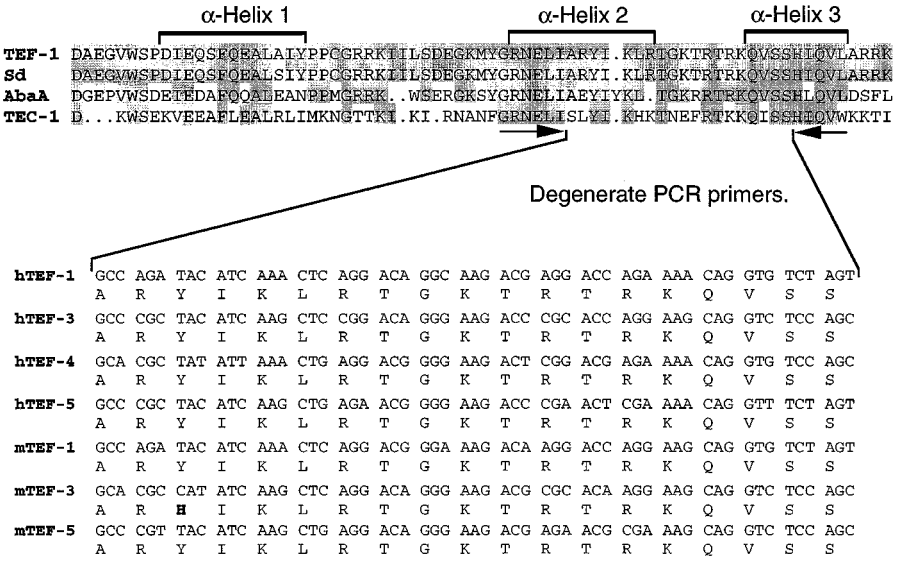

FIG. 1. **Evolutionary conservation of the TEA domain.** The *upper panel* shows the alignment of the amino acid sequences of the human TEF-1, *Drosophila scalloped* (*sd*), *Aspergillus nidulans AbaA*, and the yeast TEC1 proteins. Highly conserved residues are *boxed*. The positions of the predicted α-helices are indicated *above* the sequences. The *arrows* indicate the positions of the degenerate oligonucleotides used for PCR amplification of the novel TEA domains shown in the *lower panel*. The *lower panel* shows the nucleotide and amino acid sequences of the PCR-amplified TEA domain subregions from the novel proteins hTEF-3, hTEF-4, mTEF-3, mTEF-4, as well as the previously reported sequences for hTEF-1 and mTEF-1.

by *in vivo* excision of the λZAP II libraries using T7 or T3 RNA polymerase as appropriate. Control sense riboprobes were synthesized from the same templates again using T3 or T7 RNA polymerase as appropriate. The probes contained the entire coding region as well as the 5′ and 3′ noncoding regions. To verify that there was no cross-hybridization between probes due to the conserved TEA domain, *in situ* hybridization was performed using probes lacking the TEA domain and 5′ regions, and results similar to those shown in Figs. 7–9 were obtained (data not shown). Probe length was reduced by a 1-h alkaline lysis, and *in situ* hybridization was performed as described (48).

RESULTS

Cloning and Characterization of Novel Human and Murine Transcription Factors with TEA/ATTS Domains

The amino acid sequences of the known TEA/ATTS (hereafter TEA) domains show highest conservation in putative α-helices 2 and 3 (Fig. 1). Two degenerate oligonucleotides (see "Materials and Methods" and Fig. 1) deduced from the sequence encoding GRNELIA and the complement of the sequence encoding HIQVL were used in polymerase chain reaction (PCR) amplification experiments with cDNA libraries from either human or mouse cells. Amplification products of the expected size were cloned and their DNA sequence determined. In addition to partial cDNAs for the human and murine TEF-1 (hTEF-1 and mTEF-1 in Fig. 1), four other partial cDNAs were obtained (hTEF-3, hTEF-4, mTEF-3, and mTEF-4 in Fig. 1, hTEF-2 has already been ascribed to an unrelated protein recognizing the SV40 GT-IC enhanson. Refs 1, 49). These cDNAs encode the same amino acid sequence as TEF-1 but have a distinct nucleotide sequence, with the exception of mTEF-3 that also contains a Tyr to His substitution (in bold in Fig. 1). hTEF-1 was amplified from HeLa and human fetal brain cDNA libraries, hTEF-3 from the HeLa library, and hTEF-4 only from the human fetal brain library. The mTEFs were all amplified from a retinoic acid-differentiated embryonic stem cell library and a 10.5-day mouse embryo library.

The cDNA libraries were rescreened with the novel partial cDNAs, and full-length clones encoding hTEF-3, mTEF-3, mTEF-4, and a truncated clone encoding hTEF-4 were isolated. Alignment of the amino acid sequences of hTEF-1, hTEF-3, and hTEF-4 (Fig. 2) showed that overall hTEF-3 is 76% identical to hTEF-1, while hTEF-4 is 67% identical to hTEF-1. Nevertheless, conservation is not homogeneous throughout the protein. The TEA domains of hTEF-3 and hTEF-4 are identical to that of hTEF-1, and the carboxyl 200 amino acids are well conserved in all the hTEFs. In contrast, the N-terminal regions and the regions immediately following the TEA domain are much more divergent.

Alignment of the amino acid sequences of the mTEFs (Fig. 3*A*) showed a similar pattern with respect to the overall homology and the regions of conservation and divergence. One exception to this, however, is the TEA domain of mTEF-3 that contains five amino acid substitutions (*underlined* in Fig. 3*A*). Three of these changes, D39E, S62T, and D63E, are conservative and are at positions that are less well conserved in the TEA domains of *Aba*A and TEC1. On the other hand, the Q42R and the Y77H changes are less conservative. Moreover, Tyr-77 is invariant in all other known TEA domains (see Fig. 1).

The interspecies comparison showed that, as described previously (28, 50), mTEF-1 is 99% identical to hTEF-1. hTEF-3 is most closely related to mTEF-3. This is clearly seen by comparing the divergent regions of the proteins (see above) that are greater than 90% identical between hTEF-3 and mTEF-3 (Fig. 3*B*), whereas they are only 10–40% identical between mTEF-3 and the other h- or mTEFs (Figs. 2 and 3*A*). By the same criteria, mTEF-4 is the counterpart of hTEF-4 (see Fig. 3*B*).

Another striking feature of the amino acid sequences is that with the exception of mTEF-4, which has an extended open reading frame (ORF) upstream of the TEA domain beginning with an AUG codon, all the ORFs initiate with an AUU codon encoding isoleucine (Ref. 2, and data not shown). Analysis of the nucleotide sequences of the 5′-untranslated regions showed that in each case the ORFs are preceded by upstream stop codons and that there are no in frame AUG codons downstream of these stops (data not shown). In addition, the 5′-untranslated regions are highly divergent, and sequence homologies between family members begin only immediately upstream of the ORFs.

The TEFs Differ in Their Ability to Bind Cooperatively to Tandemly Repeated Sites

To assess the ability of the members of the TEF-1 family to bind to the GT-IIC and Sph(I + II) enhansons of the SV40 enhancer, their ORFs were cloned into the eukaryotic expression vector pXJ41 and transfected into Cos cells. The transfected cell extracts were then tested in EMSA. In extracts from cells transfected with vectors expressing hTEF-1 and hTEF-3, a specific complex (complex B) was formed with an oligonucleotide containing a single wild-type GT-IIC enhanson, whereas no complex was observed with extracts from mock-transfected cells or with a mutated GT-IIC enhanson (Fig. 4*A*, *lanes 1–6*).

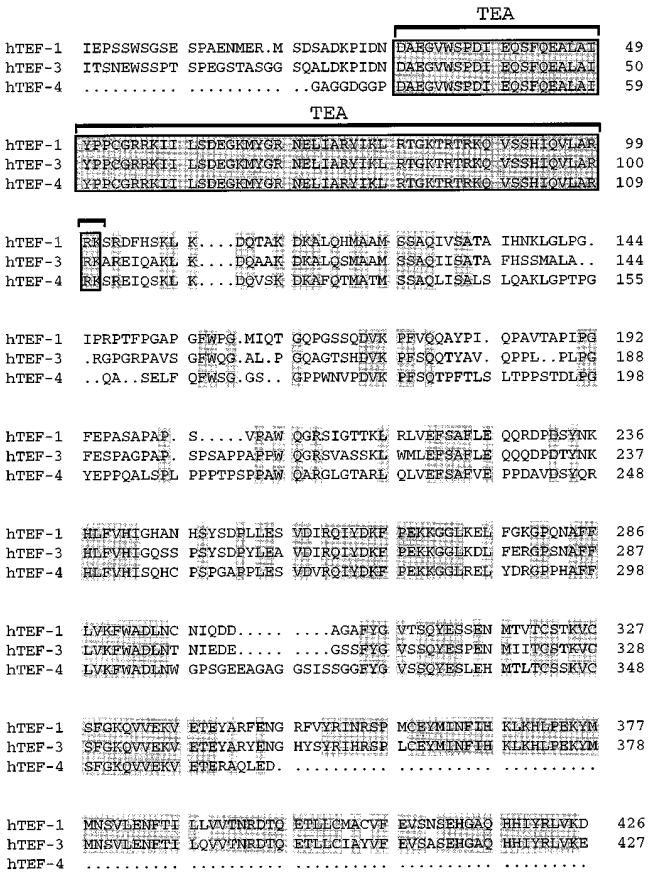

FIG. 2. **Alignment of the amino acid sequences of the hTEF proteins.** The deduced amino acid sequences of the three hTEF proteins are aligned. Amino acids conserved in all three proteins are *boxed*. In the 3′ region where no hTEF-4 sequence is available amino acids conserved in hTEF-1 and hTEF-3 are *boxed*. The position of the TEA domain is indicated. The *numbers* to the *left* indicate the amino acid coordinates. For hTEF-4 we have assumed an extended homology with mTEF-4.

hTEF-1 bound cooperatively to tandem repeats of the GT-IIC enhanson as judged by the preferential formation of complex A in which both binding sites are occupied (Fig. 4*A*, *lane 8*; Fig. 4*B*, *lane 5*; Refs. 1, 2 and see below). Note that in some lanes minor complexes *A′* and B′, formed by the binding of a truncated proteolytic degradation product of the TEFs, and complex *C*, a dimer formed between the full-length and truncated TEFs, can be observed. hTEF-1 also bound cooperatively to the tandem Sph(I + II) enhansons (Fig. 4*A*, *lane 11*, and Fig. 4*B*, *lane 9*). In contrast, formation of complex A on the tandemly repeated GT-IIC enhanson was much less efficient with hTEF-3 (compare complexes A and B formed with hTEF-1 and hTEF-3 in Fig. 4*A* and with lower amounts of extract in *panel B*) indicating that hTEF-3 binds noncooperatively. As cooperativity is required for efficient binding to the low affinity Sph enhansons (Ref. 1, and see below), binding of hTEF-3 to the Sph enhansons was much weaker than hTEF-1 (compare *Fig. 4A*, *lanes 11–12*, and Fig. 4*B*, *lanes 9* and *10*). Thus, although hTEF-3 recognizes the GT-IIC enhanson, cooperative binding to tandem repeats is impaired resulting in reduced binding to the low affinity Sph enhansons.

Similar experiments performed with increasing quantities of mTEF-3 transfected cell extracts resulted in the formation of complex B on the GT-IIC enhanson (Fig. 4*C*, *lanes 4–6*). However, when compared with hTEF-1, the formation of complex A on the tandemly repeated enhansons was much less efficient (compare *lanes 3* and *5* where there is equal formation of

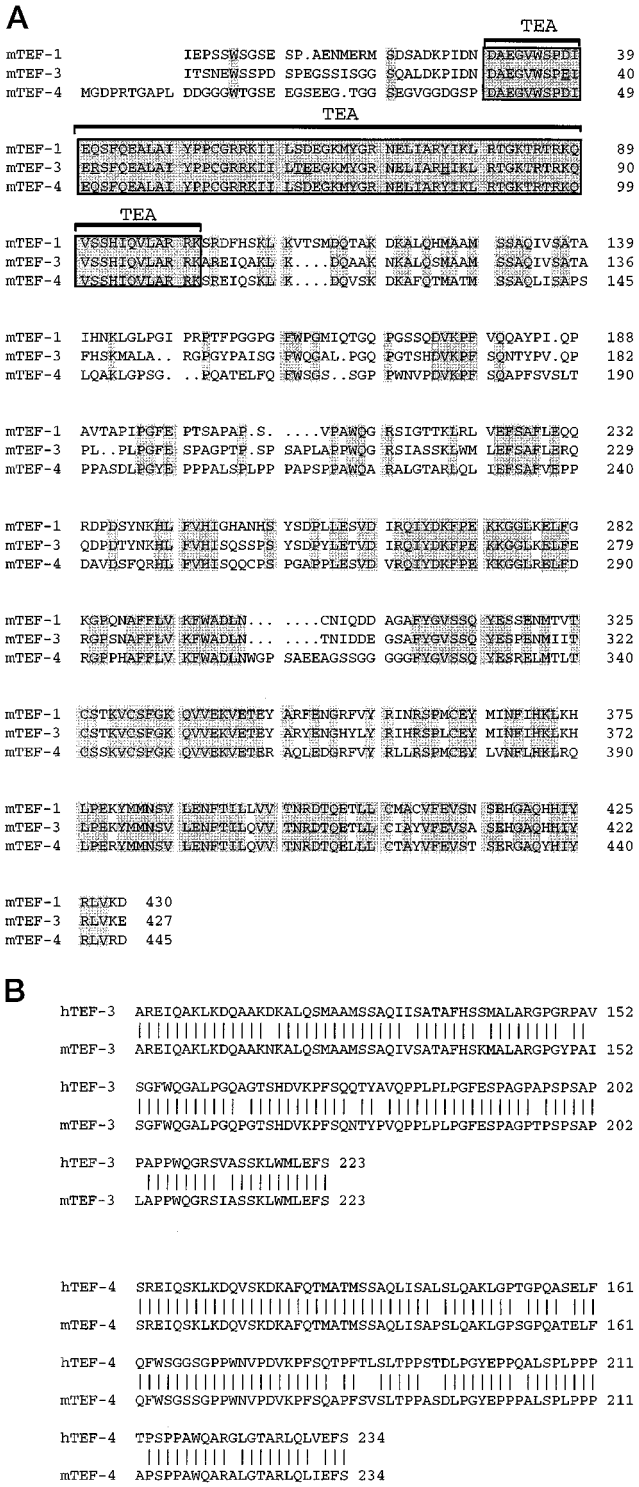

FIG. 3. *A*, alignment of the amino acid sequences of the mTEF proteins. Amino acids conserved in all three proteins are *boxed*. The position of the TEA domain is indicated. The amino acid substitutions in the TEA domain of mTEF-3 are *underlined*. *B*, comparison of the hTEF-3 and mTEF-3 and hTEF-4 and mTEF-4 amino acid sequences. Only the comparison of the divergent region following the TEA domain is shown.

complex B with *lanes 15* and *17* and *21* and *23*). Comparison of the ratio between complexes A and B shows that even at the highest concentration of mTEF-3 more of complex B than A is formed, whereas with hTEF-1 formation complex A is favored (compare *lanes 18* and *24* with *15* and *21*). These results show that, despite the five amino acid substitutions within its TEA domain, mTEF-3 binds to the GT-IIC and Sph enhansons but,

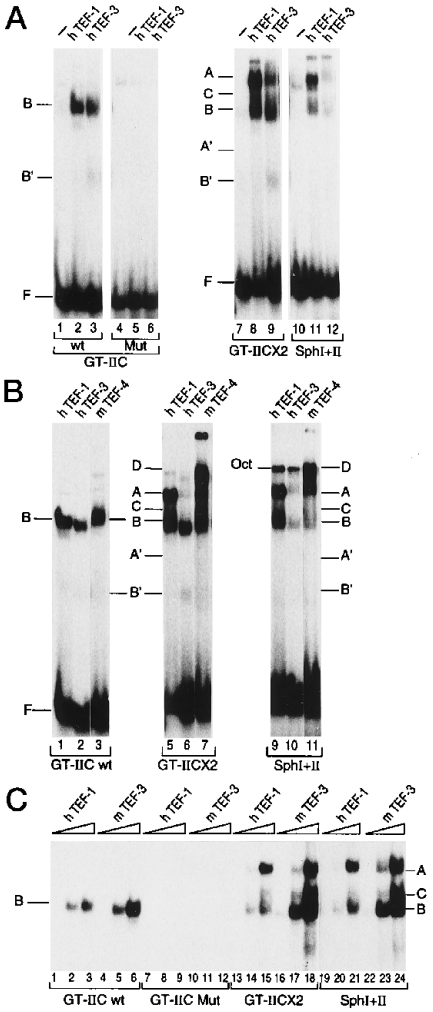

FIG. 4. *A*, autoradiography of EMSA performed with extracts from Cos cells transfected with vectors expressing hTEF proteins. The hTEF overexpressed in each cell extract is indicated *above* each lane. Reactions in *lanes 1, 4, 7,* and *10* were performed with extract from mock-transfected cells. The oligonucleotides used in each reaction are indicated *below* each lane. GT-IIC wt contains the wild-type (*wt*) enhanson, *Mut* the mutated enhanson, *GT-IICX2* the tandemly repeated enhanson, and *SphI+II* the tandemly repeated Sph enhansons from the SV40 enhancer as described previously (13). *F* indicates the position of the free oligonucleotides; *B* indicates the position of the complex formed by binding of the full-length TEF proteins to one binding site; and *A* indicates the complex formed by the simultaneous binding of protein to two binding sites. *A′* and *B′* are equivalent to *A*- and *B*-formed proteolytic degradation products of the TEFs, and *C* is the dimer formed between full-length and truncated TEFs on tandemly repeated sites. *B*, comparison of the binding of the hTEFs and mTEF-4 to the GT-IIC and Sph enhansons. The overexpressed protein is indicated *above* each lane. *A, B, C,* and *F* are as in *panel A*. *Oct* indicates the complex formed by the binding of cellular Oct-1 protein to the Oct enhanson overlapping the Sph enhansons, and *D* indicates the position of the higher order complex formed by the binding of mTEF-4. *C*, binding of mTEF-3 to the tandemly repeated GT-IIC or Sph motifs. Increasing quantities (0.5, 1.5, and 3 μl) of extract from cells transfected with the expression vectors for the TEF proteins shown *above* each lane were used in EMSA. The positions of the *A, B,* and *C*, complexes are indicated.

as with hTEF-3, cooperative binding to tandemly repeated sites is impaired.

In EMSA, mTEF-4 bound to both the GT-IIC and Sph enhansons (*lanes 3, 7,* and *11* in Fig. 4*B*). Strikingly, in addition to complexes A and B, complex D, possibly a trimer or a tetramer, was formed on tandemly repeated sites. Complex D has fortuitously the same electrophoretic mobility as the complex formed by the cellular Oct-1 factor binding to the Oct enhanson overlapping the Sph enhansons (complex *Oct* in *lanes 9–11*; and see Refs. 1, 2, 51). Complex D was the most abundant species formed with mTEF-4 indicating that its formation was highly cooperative. In addition, multimeric complexes were formed that did not enter the gel (see *top* of *lanes 7* and *11*). Thus, unlike hTEF-3 and mTEF-3 that showed a reduced ability to bind cooperatively, mTEF-4 cooperatively formed multimers on tandemly repeated binding sites.

Cooperative Binding to Tandemly Repeated Sites Is an Intrinsic Property of the TEA Domain

We next asked if the TEA domain alone would bind cooperatively to tandemly repeated sites. A 6 histidine-tagged TEA domain fusion protein (6His-TEA) was purified from *Escherichia coli* (see "Materials and Methods" and Fig. 5*A*) and used in EMSA. The 6His-TEA protein bound to both the tandemly repeated GT-IIC and Sph enhansons to generate complex B in which only one of the two sites is occupied and complex A in which both are occupied (Fig. 5*B*, *lanes 1* and *19*). Complex A was efficiently formed at the lowest concentration of fusion protein despite the presence of an excess of free oligonucleotides, indicating that binding was highly cooperative. However, complex B was formed more readily on the high affinity GT-IIC enhanson than on the low affinity Sph enhansons. These observations indicate that the TEA domain binds cooperatively to tandemly repeated GT-IIC or Sph enhansons.

EMSA was also performed with oligonucleotides containing directly repeated elements spaced by 5 or 10 nucleotides. Strikingly, compared with the efficient binding of 6His-TEA to tandemly repeated Sph enhansons, almost no binding to the spaced repeats was observed at low protein concentrations (Fig. 5*B*, *lanes 7–9* and *13–15*), confirming that cooperativity is required for binding to these low affinity enhansons. Only at high concentration of the fusion protein was formation of complex B observed, but almost no complex A was formed (*lanes 11–12* and *17–18*). Insertion of 5 or 10 nucleotides between the GT-IIC enhansons had no effect on the formation of complex B, but at low concentrations of fusion protein the formation of complex A was dramatically reduced (*lanes 24–26* and *29–31*). Complex A was observed only at high concentrations of fusion protein when the pool of free oligonucleotides was limiting (*lanes 27–28* and *32–33*). Thus, while the TEA domain binds cooperatively to tandemly repeated enhansons, binding to the spaced repeats is noncooperative. These results clearly demonstrate that the ability to bind cooperatively to tandem, but not spaced repeats, is an intrinsic property of the TEA domain.

Differential Expression of the hTEFs in Cultured Cell Lines

We next investigated the expression of the hTEF proteins in human cell lines of various origins by RT-PCR with exon- and hTEF-specific primers (see "Materials and Methods"). hTEF-3 was expressed in all the cell lines tested (Fig. 6*A*), and hTEF-1 was absent only in HepG2 hepatoma cells (Fig. 6*A*, *lane 4*). hTEF-4 had a more restricted pattern of expression, being strongly expressed only in the ovarian carcinoma cell line Ovcar-3 (Fig. 6*B*, *lane 11*), while weaker expression was detected in Intestine 407 cells, in placental JEG-3 choriocarcinoma cells, and in embryonic kidney 293 cells (Fig. 6*B*, *lanes 5, 10,* and *6*, respectively). In contrast to hTEF-1 and hTEF-3, no hTEF-4 expression was detected in HeLa, HepG2, Molt 4, IMR-32, and CaCo2 cells (Fig. 6*B*, *lanes 2–4, 9,* and *12*). These results show that the hTEF proteins have distinct expression patterns in cultured cells.

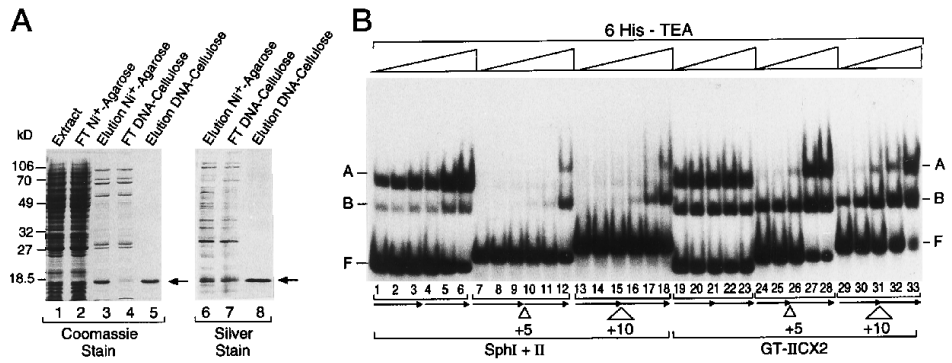

FIG. 5. *A*, purification of the histidine-tagged hTEF-1 TEA domain fusion protein. *Lane 1* shows the starting bacterial extract, *lane 2* the flow-through (*FT*) fraction from the nickel-agarose chromatography, *lane 3* the fraction eluted from the nickel-agarose column with 0.25 M immidazole, *lane 4* the flow-through fraction from the double-stranded DNA-cellulose column, and *lane 5* the 0.8 M KCl eluate from the DNA-cellulose. The gel was stained with Coomassie Blue. *Lanes 6–8* show an aliquot of the same material as in *lanes 3–5* stained with silver nitrate. The positions of prestained molecular size markers are indicated to the *left* of the panel. The *arrow* to the *right* indicates the purified 6 His-TEA protein. *B*, binding of the purified 6 His-TEA protein to tandemly repeated and spaced binding sites. *A*, *B*, and *F* are as described in Fig. 4. The tandem repeats are schematized by the *arrows*, and the presence of a 5- or 10-nucleotide spacer is indicated *below* the *arrows*. Each series of reactions shows increasing concentrations of protein from 5–50 ng.

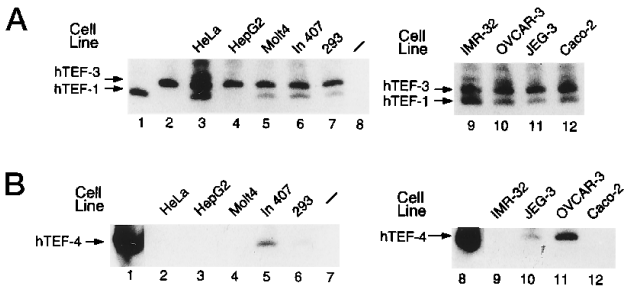

FIG. 6. *A*, expression of hTEF-1 and hTEF-3 in human cell lines. RT-PCR was used to amplify fragments of the hTEF-1 and hTEF-3 mRNAs. After electrophoresis on a 6% acrylamide gel, an aliquot of the PCR reactions was transferred to nitrocellulose and hybridized with the homologous TEA domain probe. *Lanes 1* and *2* show the PCR fragments generated using 10 pg of the corresponding expression vectors as template and *lane 8* the PCR performed in the absence of added template. The source of the RNA used in the other reactions is indicated *above* each lane. HeLa cells are derived from a cervical carcinoma, *HepG2* from a hepatocarcinoma, *Molt4* from a T-cell leukemia, *IMR32* from a neuroblastoma, *OVCAR-3* from a ovary adenocarcinoma, *JEG-3* from a choriocarcinoma, *CaCo-2* from a colon adenocarcinoma. Intestine (*In*) 407 cells are from embryonic intestine and *293* cells are transformed embryonal kidney cells. *B*, expression of hTEF-4 in human cell lines. *Lanes 1* and *8* show the amplification product generated using 10 pg of the corresponding expression vectors as templates and *lane 7* with no added template. The cell lines used for the other reactions are indicated *above* each lane.

Differential Expression of the mTEFs during Mouse Embryogenesis

As indicated by their presence in cDNA libraries from 10.5-day embryos, all the mTEFs should be expressed during embryogenesis. The expression patterns of mTEF-3 and mTEF-4 were therefore investigated and compared with that of mTEF-1 by *in situ* hybridization of mouse embryos and fetuses from 6.5 to 18.5 days post-coitum (dpc).

Early Gestational Stages (6.5 to 8.5 dpc)—All three TEFs were differentially expressed during early development in embryonic as well as extra-embryonic and maternal cell lineages. In the maternal decidua, mTEF-3 and mTEF-4 expression was somewhat complementary at 6.5 and 7.5 dpc. mTEF-3 transcripts were abundant in the embryonic (antimesometrial) pole and decreased gradually toward the ectoplacental (mesometrial) pole (Fig. 7, *C* and *G*). In contrast, weaker expression of mTEF-4, restricted to the ectoplacental pole of the outer decidua, was observed. mTEF-1 was also expressed in decidual cells (Fig. 7, *B*, *F*, and *H*), but at a lower level than mTEF-3. At 8.5 dpc, mTEF-3 was strongly expressed throughout the decidua, although dispersed cells or cell clones were unlabeled (Fig. 7*K*).

Distinct expression patterns of the mTEFs were also observed in the early conceptus. At 6.5 dpc (pregastrula, egg-cylinder stage), mTEF-1 and mTEF-3 were expressed in distinct extra-embryonic regions, mTEF-1 in the ectoplacental cone and mTEF-3 in the extra-embryonic layers (Fig. 7, *B* and *C*). Nevertheless, at 7.5 dpc (early gastrula stage), mTEF-1 expression was observed in the embryo as well as in the extra-embryonic tissues, where particularly intense signals were observed in some ectoplacental cells (Fig. 7*F*). At 8.5 dpc mTEF-1 was expressed in the entire embryo (Fig. 7*J*). At 7.5–8.5 dpc mTEF-3 was expressed at low levels in the entire conceptus (Fig. 7, *G* and *K*). In contrast, mTEF-4 was strongly expressed in the entire conceptus at 6.5 dpc (Fig. 7*D*) and remained strongly expressed in the embryo until 8.5 dpc, whereas its expression in the extra-embryonic region progressively disappeared (Fig. 7, *D*, *H*, *L*).

Mid-gestational Stages (9.5–12.5 dpc)—Both mTEF-1 and mTEF-4 were ubiquitously expressed in 9.5 dpc embryos, but from 10.5 dpc these two genes showed preferential expression in specific tissues, some being common to both genes. Both mTEF-1 and mTEF-4 were strongly expressed in the ventricular layer of the neuroepithelium that contains the mitotic neuroblasts. This was observed both in the developing brain and spinal cord (compare Fig. 8, *B*, *D*, and *F*, *H*). Strikingly, no labeling was observed in the surrounding mantle layer that contains post-mitotic neural precursors. In contrast, the distribution of mTEF-1 and mTEF-4 transcripts differed in various mesenchymes, for example the facial and gut mesenchymes where accentuated mTEF-4 expression was observed (Fig. 8, *F* and *H*). On the other hand, mTEF-1, but not mTEF-4, was preferentially expressed in the developing myocardium as early as 10.5 dpc (Fig. 8*B*). Note also that the heart chambers, the cavities of the outflow tract, and the descending aorta were unlabeled by all mTEF probes suggesting that these genes are not expressed in embryonic blood. At later stages strong mTEF-1 expression was also seen in various muscle anlagen, both in facial and at other axial levels (Fig. 8*J*).

Strikingly, mTEF-3 signals were only detected in the developing myotomes (the myogenic compartment of the somitic mesoderm) and appeared in the cranio-caudal progression from cervical levels (at 9.5 dpc, data not shown) to trunk and caudal levels (10.5 dpc, Fig. 8*D*). Indeed, skeletal muscle precursors were specifically labeled by the mTEF-3 probe at 11.5–12.5 dpc

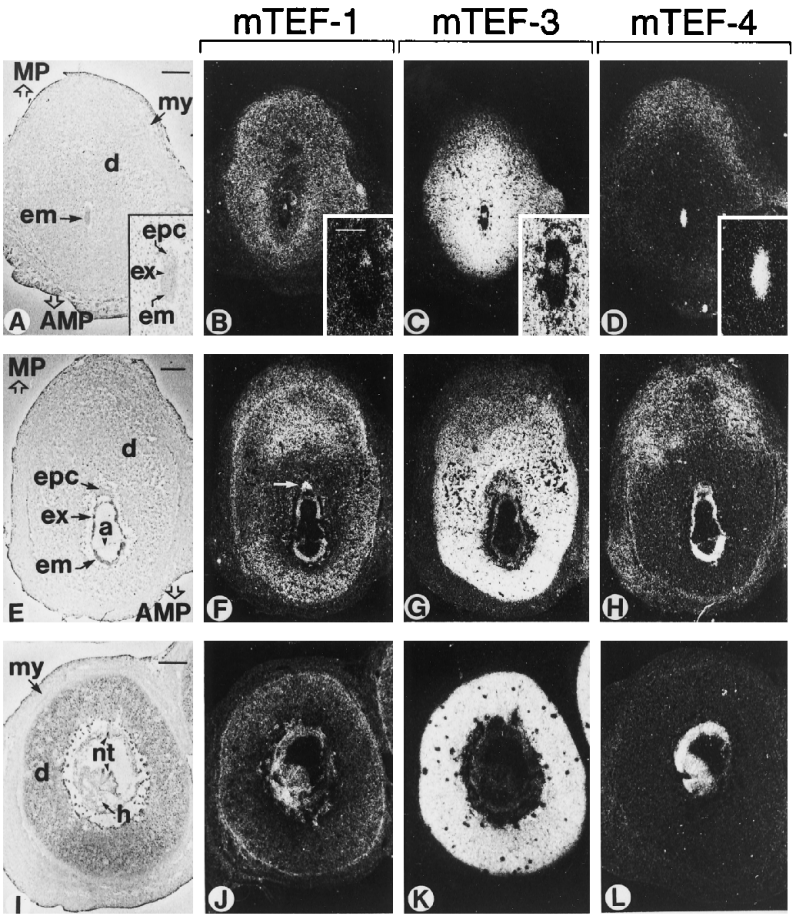

FIG. 7. **Expression of mTEFs at 6.5–8.5 dpc.** *A–D*, three serial sections through a 6.5 dpc (egg cylinder stage) mouse embryo sectioned *in utero*. As in the following figures, the *left-hand side panel* is a bright-field view of the section showing the histology, and the *right-hand side panels* are dark-field views of sections hybridized, respectively, to mTEF-1 (*B*), mTEF-3 (*C*) and mTEF-4 (*D*) riboprobes. Dark-field views reveal the hybridization signal grain in *white*. *Scale bar*, 450 μm. The *insets* show high power magnifications of the conceptus. *Scale bar*, 160 μm. *E–H*, serial sections through a 7.5 dpc (primitive streak stage) embryo sectioned *in utero*. The *arrow* in (*F*) points to the strong labeling of mTEF-1 in cells of the ectoplacental cone. *Scale bar*, 600 μm. *I–L*, serial sections of a 8.5 dpc embryo (early somite stage, prior to embryo turning) sectioned *in utero*. These sections cross the developing heart and trunk region, as well as more lateral and caudal regions of the embryo toward the *upper left side* of the cavity. *Scale bar*, 750 μm. Abbreviations: *a*, amniotic membrane; *AMP*, anti-mesometrial pole; *d*, decidual tissue; *em*, embryonic germ layers; *epc*, ectoplacental cone; *ex*, extra-embryonic layers; *h*, heart; *MP*, mesometrial pole; *my*, myometrium; *nt*, neural tube.

including head, axial, body wall, and limb muscle anlagen (Fig. 8*G*, and data not shown). The developing myocardium was never labeled at any stage by the mTEF-3 probe (Fig. 8*D*, and data not shown).

Late Developmental Stages (13.5–18.5 dpc)—In skeletal muscle, mTEF-3 expression persisted during late gestational stages (Fig. 8*K* shows the head, neck, shoulder, intercostal, abdominal wall, and hindlimb muscles). Interestingly, the mTEF-3 probe yielded a "spotted" labeling in 15.5 dpc and older developing muscles, some of the cells showing much more intense labeling (illustrated in Fig. 9*C* for the shoulder muscles). Nevertheless, mTEF-3 transcripts were detected in several discrete regions outside the developing muscle from 15.5 to 18.5 dpc, namely the liver, the lung, the salivary gland, and nasal gland epithelia, and the small intestine presumably in the duodenal region (Fig. 8*K* and data not shown). mTEF-1 expression also persisted in late developing muscles where the labeling was more homogeneous than that of mTEF-3 (compare Fig. 8, *J–K*, and Fig. 9, *B* and *C*). As at earlier stages the mTEF-1 signal remained high in the differentiating myocardium (data not shown).

At later stages, strong expression of mTEF-1 and mTEF-4 persisted in the ventricular zone of the CNS (see the 15.5-dpc forebrain ventricles in Fig. 8, *J* and *K*) that tends to become thinner as development proceeds. Both genes also showed accentuated expression in the developing lungs at 15.5 dpc (Fig. 8, *J* and *L*) and at later stages (not shown). However, distinct mTEF-1 and mTEF-4 expression patterns were observed in a number of developing organs or tissues, some examples of which are illustrated. mTEF-1 was strongly expressed in the entire nasal epithelium (both in the olfactory and respiratory regions) as well as in the surrounding mesenchyme (Fig. 9*E*; note that mTEF-1 transcripts appear more abundant toward the apical layers of the olfactory epithelium). In contrast, mTEF-4 transcripts were clearly more abundant in the basal cell layer of the olfactory epithelium (Fig. 9*F*). mTEF-1 was rather uniformly expressed in the developing kidney (metanephros), whereas accentuated mTEF-4 expression was restricted to the cortical region corresponding to the nephrogenic zone where new nephrons are being generated (Fig. 9, *G–I*; note also the higher expression of mTEF-1 in the adrenal gland).

As first observed at 12.5 dpc, mTEF-4 was strongly expressed in the mesenchyme of the intestinal loops, whereas lower mTEF-1 expression was observed in both the mesenchymal and epithelial components (Fig. 9, *J–L*). Pronounced mTEF-1 expression was detected in the most internal layer of the urinary bladder epithelium as well as in the external layer of the mesenchyme, whereas mTEF-4 transcripts were more evenly distributed in the entire bladder mesenchyme and epithelium (Fig. 9, *J–L*). Interestingly, specific expression of mTEF-4 along the lining of some hepatic blood vessels was observed (Fig. 9*L*). As no such endothelial labeling was detected in other blood vessels outside the liver, it appears specific to the portal system.

DISCUSSION

A Novel Family of Transcription Factors Sharing a Common DNA Binding Domain, but with Differential DNA Binding Properties—We report here the molecular cloning of four novel mammalian members of the TEA domain family of transcription factors with extensive homology to TEF-1, notably in the TEA domain and in the carboxyl 200 amino acids. Several regions of TEF-1 have distinctive amino acid compositions often shared in other transcription factors, a proline-rich region between amino acids 143 and 204, a region rich in serine, threonine, and tyrosine between amino acids 306 and 328, and

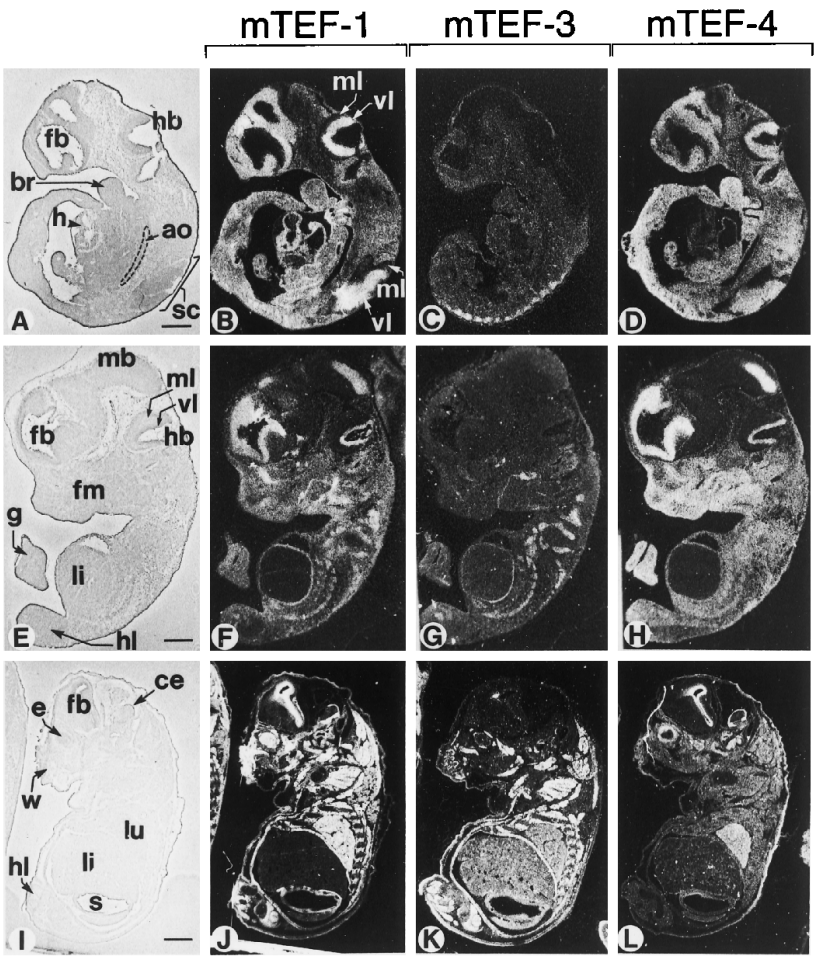

FIG. 8. **Expression of m-TEFs at 10.5–15.5 dpc.** *A–D*, sagittal sections of a 10.5 dpc embryo, hybridized as indicated to mTEF-1 (*B*), mTEF-3 (*C*), and mTEF-4 (*D*) riboprobes. *A–B* and *D* are two adjacent sections that cross the body axis slightly laterally to the midline. In these sections, the spinal cord is sectioned only in the trunk region due to skewing of the embryo axis. Section *C* is a more lateral plane that shows the mTEF-3 segmented labeling pattern in presumptive myotomes. *Scale bar*, 600 μm. *E–H*, serial sections through the lateral regions of the head and trunk of a 12.5 dpc embryo. *Scale bar*, 750 μm. *I–L*, serial sections through the lateral regions of the head and trunk of a 15.5 dpc embryo. *Scale bar*, 1.5 mm. Abbreviations: *ao*, aorta; *br*, (first) branchial arch; *ce*, cerebellum; *e*, eye; *fb*, forebrain; *fm*, facial mesenchyme; *g*, herniated gut; *h*, heart; *hb*, hindbrain; *hl*, hindlimb; *li*, liver; *lu*, lung; *mb*, midbrain, *ml*,: mantle layer; *s*, stomach; *sc*, spinal cord, *vl*, ventricular layer; *w*, whisker follicles.

a region with the potential to form a zinc finger at the extreme C terminus (2). We have subsequently shown that these three regions are involved in transcriptional activation, although the potential of the carboxyl region to form a zinc finger was not required for transactivation (13). Comparison of the amino acid sequences of the TEF proteins shows that, although each contains a proline-rich region, its primary amino acid sequence is among the least well conserved. The serine, threonine, and tyrosine-rich region is well conserved; however, despite the overall high homology in the carboxyl regions, the cysteine residues in the putative zinc finger are not all conserved. We have attempted to compare the transcriptional properties of the TEFs by transfection in HeLa cells. However, in agreement with the high conservation in the regions involved in transactivation, each TEF had a dominant negative phenotype[2] due to a transcriptional interference/squelching effect as observed with TEF-1 (2, 13).

We determined the relationship between TEF-3/4 and other previously described TEA domain proteins. For *Aba*A and TEC1 the sequence similarities outside the TEA domain were too low to allow any relationships to be determined. However, *sd* was more related to TEF-1 than to the other TEFs.[2] Strikingly, cTEF-1/RTEF-1 (32, 52) is 90% identical to hTEF-3 but only 77% identical to hTEF-1. Thus, the cTEF isolated by Stewart *et al.* (32) should be considered as a counterpart of TEF-3 rather than of TEF-1, whose avian counterpart has been designated NTEF-1 (52). mTEF-4 is clearly identical to ETF (11) that was reported to be specifically expressed in the developing brain. Although our results also show strong expression of mTEF-4/ETF in mitotic neuroblasts, it is clearly expressed in other tissues (see below). While this manuscript was in preparation, an additional chicken TEF gene, DTEF-1, was described (52). We have also isolated a human cDNA (hTEF-5) homologous to DTEF-1 and are presently characterizing its properties.[2]

As expected from the fact that the TEF proteins share a common DNA binding domain, each binds to the GT-IIC and Sph enhansons from the SV40 enhancer. This is true even of mTEF-3 whose TEA domain contains five amino acid substitutions one of which changes a highly conserved Tyr residue. However, our results do not exclude the possibility that the amino acid changes in the mTEF-3 TEA domain may confer the ability to recognize additional unrelated sequences.

Although all the TEF proteins bind to the previously defined consensus binding site exemplified by the SV40 GT-IIC and Sph enhansons, they exhibit differences in their ability to bind cooperatively to tandemly repeated sites. mTEF-4 not only binds cooperatively to tandemly repeated sites, but it cooperatively generates higher order complexes, probably trimers or tetramers. In contrast, h- and mTEF-3 bind essentially noncooperatively. Consequently, binding of these proteins to the low affinity Sph sites is inefficient when compared with hTEF-1 or mTEF-4. Although the changes within the TEA domain of mTEF-3 may contribute to the diminished cooperativity, this cannot be the case for hTEF-3 whose TEA domain is identical to that of TEF-1. Moreover, as the TEA domain alone binds cooperatively to tandemly repeated sites, the above variations must result from the differential abilities of other regions of the TEFs to either decrease or enhance cooperativity. Thus, al-

[2] P. Jacquemin, J.-J. Hwang, J. A. Martial, P. Dollé, and I. Davidson, unpublished data.

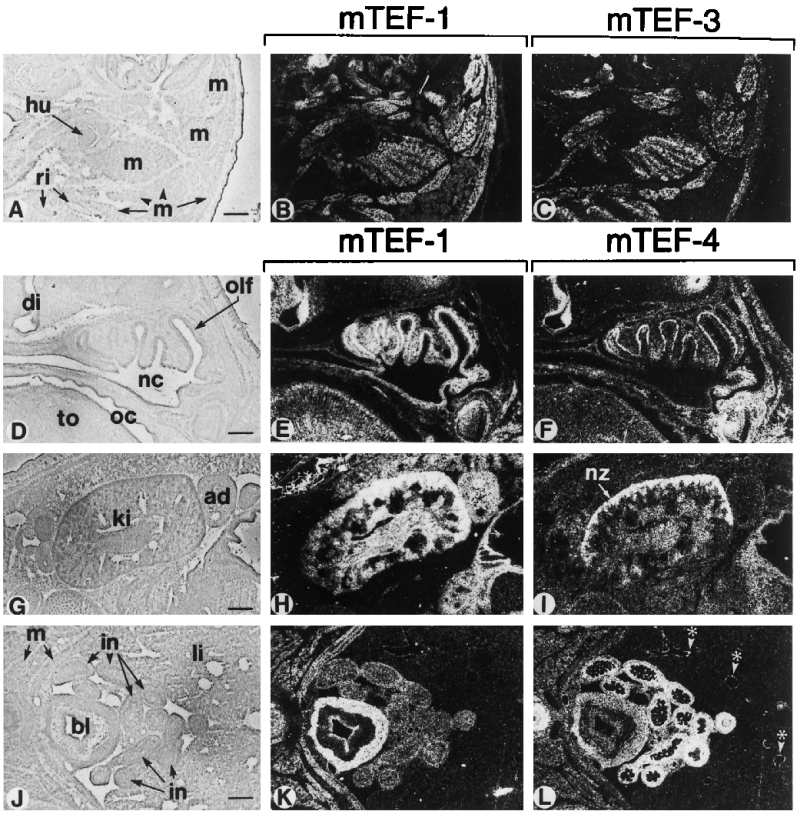

FIG. 9. **Differential expression of mTEFs during late gestation.** *A–C*, sagittal section through the shoulder region of a 15.5 dpc fetus. These views illustrate mTEF-1 and mTEF-3 labeling patterns in the shoulder, cervical, and upper intercostal muscles (not all of which are indicated). *Scale bar*, 600 μm. *D–F*, sagittal sections crossing the olfactory epithelium of a 16.5 dpc fetus, hybridized to mTEF-1 and mTEF-4 probes, respectively. *Scale bar*, 500 μm. *G–I*, sagittal sections through the kidney and adrenal gland of a 16.5 dpc fetus. *Scale bar*, 500 μm. *J–L*, coronal sections through the lower abdominal cavity and urinary bladder of a 16.5 dpc fetus. The *asterisks* point to mTEF-4 labeling in the endothelium of hepatic blood vessels. *Scale bar*, 500 μm. Abbreviations: *ad*, adrenal gland; *bl*, urinary bladder; *di*, diencephalon; *hu*, humerus; *in*, intestine; *ki*, kidney; *m*, muscles (non-exhaustive); *nc*, nasal cavity; *nz*, nephrogenic zone; *oc*, oral cavity; *olf*, olfactory region; *li*, liver; *ri*, ribs; *to*, tongue.

though the TEA domain is the minimal domain required for specific and cooperative DNA binding, its activity is modulated by other regions of the TEF proteins. Such a result is not without precedent as a mechanism for intramolecular modulation of DBD activity has been described in the Ets-1 factor (53, 54).

The mTEFs Are Differentially Expressed during Early Embryonic Development—The mTEFs are differentially expressed in the early conceptus at 6.5 dpc. mTEF-1 and mTEF-3 were specifically expressed in the extra-embryonic tissues, notably a strong expression of mTEF-1 in the ectoplacental cone, whereas mTEF-4 was expressed in both the embryonic and extraembryonic tissues. The extraembryonic tissues are derived from the primitive endoderm and trophectoderm lineages that are the first to form in mammalian embryos (reviewed in Ref. 55). Consequently, our present observations suggest that, although no relevant target genes have been described, members of the TEF family are expressed early in embryogenesis (see also Refs. 1, 2, 12, 14, 38, 56, 57, reviewed in 58) and may play a role in specification of the extra-embryonic lineages.

While analyzing the expression of the TEF factors in early post-implantation embryos, we also noted differential expression of the TEFs in the maternal decidua where a strong mTEF-3 expression was observed. As mTEF-3 was not expressed in the surrounding myometrium, it is possible that mTEF-3 expression is directly related to the decidualization reaction. Further identification of the TEF-3-regulated genes will be required to understand its role in the decidua.

At Mid-gestational Stages mTEF-3 Expression Is Largely Restricted to Developing Skeletal Muscle—As described in the Introduction TEF-1 and/or related factors have been implicated in muscle-specific gene expression and cardiogenesis. The importance of the TEF factors in these processes is further supported by the results presented here. From 10.5 dpc, mTEF-1 was expressed in the developing myocardium and in skeletal muscle precursors. Even more strikingly, mTEF-3 was specifically expressed in skeletal muscle precursors as early as 9.5 dpc, but not in the myocardium. mTEF-3 and mTEF-1 were expressed in the developing skeletal muscles derived from epaxial and hypaxial lineages (59) as well as the head muscles derived from the nonovertly segmented paraxial mesoderm. The expression pattern of mTEF-3 is therefore similar, but not identical, to that of other myogenic factors, such as MyoD in both its specificity and onset of expression (reviewed in Refs. 60 and 61). The expression of mTEF-1 and mTEF-3 in muscle is maintained at late stages of embryogenesis where a heterogeneity of mTEF-3, but not mTEF-1, expression is seen, beginning around 15.5 dpc. Interestingly, this corresponds to the time at which muscle innervation and fiber type differentiation begin suggesting that mTEF-3 may be differentially expressed in different fiber types.

The expression of mTEF-3 is similar, but not identical, to that reported for its avian homologue RTEF-1, although preferential expression of cTEF-3/RTEF-1 was observed in skeletal muscle, and, unlike mTEF-3, cTEF-3/RTEF-1 was also expressed in both fetal and adult heart (32). Further studies will be required to determine whether mTEF-3 is expressed in the adult heart. However, our present results support those of Farrance and Ordahl (62) who indicated a key role for cTEF-3/RTEF-1 in muscle-specific gene transcription in chicken. Thus, while previous studies on myogenesis have concentrated on the MyoD and MEF2 families of factors (63), the correlation between the expression of mTEF-1 and mTEF-3 and that of known muscle-specific target genes provides strong evidence that these TEF factors are also likely to be important for myogenesis.

The Overlapping Expression Zones of the mTEFs Suggest Partially Redundant Roles in Several Developmental Processes—The expression of mTEF-1 is clearly not limited to muscle tissue but is not ubiquitous. Strong expression was observed in mitotic neuroblasts as well as in a number of developing organs. In many, but not all of these expression zones, accentu-

ated expression of mTEF-4 was also detected. Despite the fact that mTEF-1 is expressed at early stages of embryogenesis and is subsequently expressed in several tissues, the only major defects in mTEF-1 null mice concerned cardiogenesis, skeletal muscle and the central nervous system being apparently unaffected (38). As all the mTEFs bind the same consensus site, the restricted phenotype of the TEF-1 null mice may potentially be explained by the overlapping mTEF expression zones presented here. In the early conceptus and in the CNS, lack of expression of mTEF-1 may be compensated by the expression of mTEF-3 and/or mTEF-4. In skeletal muscle its expression may be compensated by expression of mTEF-3. Such redundancy has been previously noted for other myogenic factors, such as myf-5 and MyoD (Ref. 64 and references therein). However, as the lack of mTEF-1 in the developing myocardium is not compensated by expression of other mTEFs, TEF-1 null embryos die at 11.5–12.5 dpc, and their premature death prevents investigation of mTEF-1 function(s) at later stages.

In addition to zones of expression overlapping with mTEF-1, there are several regions where mTEF-4 is the predominantly expressed member of the family. mTEF-4 is strongly expressed throughout the 6.5 dpc embryo and remains so until 9.5–10.5 dpc, after which a more specific pattern of expression is established. Later in development mTEF-4 may play a role in organogenesis. For example, accentuated mTEF-4 expression was observed in the intestinal mesenchyme and the nephrogenic region of the kidney where it may contribute to the mesenchyme-epithelial transition during nephron development. The identification of TEF-regulated genes in the CNS and the above organs will help to clarify the function of the TEFs in these tissues.

mTEF-4/ETF was initially reported to be a neural-specific factor based on the results of whole mount *in situ* hybridizations and Northern blots (11). Our results, using *in situ* hybridization on sectioned embryos, more precisely define the mTEF-4/ETF expression zone to the mitotic neuroblasts. Nevertheless, mTEF-4/ETF is not a neural-specific factor since, as described above, it is strongly expressed in the embryo from 6.5 to 8.5 days, and at later times it is also strongly expressed in several other non-neural tissues.

It is interesting to compare the specific expression of the mTEFs during embryogenesis with the expression pattern of the human TEFs seen in cultured cell lines. Consistent with the fact that mTEF-1 is widely expressed in embryogenesis, but is not expressed in liver, hTEF-1 is expressed in all of the cell lines tested with the exception of the HepG2 hepatocarcinoma cells. In contrast, it is striking that hTEF-3 expression is much wider than would have been expected from observation of mTEF-3 in the mouse embryo. Analogous results have been seen with myogenic MEF2 factors that are specifically expressed during embryogenesis but are ubiquitously expressed after birth and in cultured cell lines (Refs. 65, 66 and references therein). Consistent with the observation that mTEF-4 was expressed in neural tissue, hTEF-4 was cloned from a human fetal brain library, but it was not expressed in IMR32 neuroblastoma cells. Similarly, mTEF-4 was expressed in the developing gut mesenchyme, and hTEF-4 was expressed Intestine 407 cells derived from embryonic intestine.

Our results imply that the TEF family of transcription factors may be involved in several developmental processes from early times of embryogenesis. Further gene targeting experiments will help to define more precisely the functions of these factors.

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