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Human TEF-5 Is Preferentially Expressed in Placenta and Binds to Multiple Functional Elements of the Human Chorionic Somatomammotropin-B Gene Enhancer*

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We report the cloning of a cDNA encoding the human transcription factor hTEF-5, containing the TEA/ATTS DNA binding domain and related to the TEF family of transcription factors. hTEF-5 is expressed in skeletal and cardiac muscle, but the strongest expression is observed in the placenta and in placenta-derived JEG-3 choriocarcinoma cells. In correlation with its placental expression, we show that hTEF-5 binds to several functional enhansons of the human chorionic somatomammotropin (hCS)-B gene enhancer. We define a novel functional element in this enhancer comprising tandemly repeated sites to which hTEF-5 binds cooperatively. In the corresponding region of the hCS-A enhancer, which is known to be inactive, this element is inactivated by a naturally occurring single base mutation that disrupts hTEF-5 binding. We further show that the binding of the previously described placental protein f/chorionic somatomammotropin enhancer factor-1 to TEF-binding sites is disrupted by monoclonal antibodies directed against the TEA domain and that this factor is a proteolytic degradation product of the TEF factors. These results strongly suggest that hTEF-5 regulates the activity of the hCS-B gene enhancer.

Human transcriptional enhancer factor (hTEF)-1[1] is the prototype member of the family of transcription factors containing the TEA/ATTS (hereafter called TEA) DNA binding domain (DBD, Refs. 1–3). Transcription factors belonging to this family have been identified in several organisms, where they fulfill various developmental functions. For instance, in yeast the TEC1 protein is postulated to regulate transcription from the Ty1 transposon and is required for pseudohyphal growth (4–6), while in *Aspergillus nidulans* the AbaA factor controls a regulatory circuit in the terminal stages of conidiophore development (7, 8). The *Drosophila scalloped* gene is required for normal development of the central and peripheral nervous systems, taste behavior, and normal wing morphology (9, 10).

In chicks and in mammals, binding sites for TEA domain proteins have been described in diverse types of enhancers with different tissue specificities (Refs. 1 and 11–18 and references therein, and see below). The activity of these sites was originally attributed to the binding of TEF-1, the first cloned mammalian TEF factor, identified by its binding to the GT-IIC and Sph enhansons of the simian virus 40 (SV40) enhancer, where it regulates transcription from the early and late promoters (1, 19–26). TEF-1 is expressed widely, but not ubiquitously, from early stages of murine embryonic development and in many established cell lines (Refs. 27–29 and references therein). TEF-1 expression is particularly pronounced in developing skeletal and cardiac muscle and in mitotic neuroblasts. Despite this wide pattern of expression, TEF-1 null mice show defects only in the heart, leading to embryonic lethality (28).

The vertebrate genome encodes at least four related TEF factors with the TEA DBD (TEF-1, -3, -4, and -5; Refs. 13, 28, and 30–35), all of which bind to the consensus site (5′-(A/T)(A/G)(A/G)(A/T)ATG(C/T)(G/A)-3′), containing a conserved ATG core. The TEF-3 factor (28), also called chick RTEF-1 (35) or mouse TEFR1/FR-19 (Refs. 31 and 32; summarized in Table I) is expressed in several cell lines, and its expression can be induced by mitogenic stimulation of quiescent fibroblasts or by *in vitro* differentiation of myoblasts to myotubes (28, 31, 32). In contrast to TEF-1, the expression of TEF-3 during mouse embryonic development is largely restricted to the skeletal muscle lineage, where it can be clearly seen at 10.5 days postcoitum, although at later times it is also expressed in the developing lung and liver (28). In adult mice and chicken, TEF-3 is expressed also in cardiac muscle (31–33, 35). In addition to TEF-1 and TEF-3, DTEF-1, whose expression is also enriched in cardiac muscle, has been described in chicken (Ref. 33; see Table I). The muscle-enriched expression of these TEFs correlates with the expression of known target genes, such as α- and β-myosin heavy chain, α-skeletal actin, and cardiac troponin C, whose enhancers contain the TEF-binding M-CAT motif (11–17, 36–41), pointing to a potential role for these TEFs in skeletal and cardiac muscle development.

TEF-4 was first described as a neuron-specific factor in the mouse as ETF (Ref. 34; see Table I); however, we have shown that it is strongly expressed throughout the embryo as early as 6.5 days postcoitum, while at later times its expression becomes more restricted to mitotic neuroblasts and to various mesenchymes (28). At later stages of embryogenesis, TEF-4 is

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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) X94439.

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[1] The abbreviations used are: hTEF, human TEF; mTEF, mouse TEF; hCS, human chorionic somatomammotropin; CSEF-1, chorionic somatomammotropin enhancer factor-1; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; mAb, monoclonal antibody; PPf, placental protein f; WCE, whole cell extract(s); DBD, DNA-binding domain.

TABLE I
Nomenclature of mammalian and avian TEA domain proteins

A comparison of vertebrate TEF factors is shown. The murine and chicken homologues of the human TEFs are indicated (h- homologues are human, m- are mouse, and c- are chicken). ND indicates that at present no chicken homologue of TEF-4 has as yet been described. The numbers in parentheses indicate the references for each factor.

	TEF-1	TEF-3	TEF-4	TEF-5
Human	hTEF-1 (1)	hTEF-3 (28)	hTEF-4 (28)	hTEF-5[a]
Mouse	mTEF-1 (30)	mTEF-3 (28) FR-19 (31) TEFR1 (32)	mTEF-4 (28) mETF (34)	mTEF-5[b]
Chicken	cNTEF-1 (33)	cRTEF-1 (35)	ND	cDTEF-1 (33)

[a] This study.
[b] Our unpublished data.

also expressed in a number of developing organs (*e.g.* in the nephrogenic region of the kidney). Thus, the TEF-4 expression pattern is distinct from that of TEF-3 but partially overlaps with that of TEF-1. Although no target genes for the TEF factors have been described in neural and mesenchymal tissues, these observations suggest that TEF-1 and TEF-4 may play a role in neurogenesis and in the development of several organs. Thus, considered together, the above results suggest that the TEFs may play partially redundant roles in several developmental processes.

In addition to muscle-specific enhancers, putative TEF-binding sites have been noted in the placenta-specific human chorionic somatomammotropin (hCS; also called placental lactogen) B gene enhancer (42–45). The hCS-B enhancer is active in the cytotrophoblast-derived JEG-3 cell line and is progressively activated during the differentiation of primary cytotrophoblasts to syncytiotrophoblast *in vitro* (46). This enhancer can be divided into two functional elements, DF-3 and DF-4, each of which have been postulated to contain TEF-binding sites. Point mutations affecting the putative TEF-binding site within DF-4 inactivate this element (46–48). The TEF-binding site in DF-4 is recognized by placental protein f (PPf) or chorionic somatomammotropin enhancer factor-1 (CSEF-1) (43, 46–48). This factor(s) has not yet been identified, but it is apparently unrelated to TEF-1 (48).

By analogy to myogenesis, where muscle-specific TEF factors and target genes have been identified, a TEF factor(s) contributing to the function of placenta-specific enhancers may also exist. Here we report the cloning of hTEF-5, which is homologous to the B isoform of chicken DTEF-1 (33) and is expressed mainly in skeletal muscle and placenta. In correlation with this restricted expression pattern, we show that hTEF-5 binds to the M-CAT motifs of several muscle genes and to the TEF-binding site in the hCS-B DF-4 element. Furthermore, we have characterized a novel functional enhanson within the hCS-B DF-3 element composed of tandemly repeated binding sites to which hTEF-5 binds cooperatively. In the DF-3 element of the hCS-A enhancer, which is inactive in JEG-3 cells and syncytiotrophoblast, this enhanson is mutated by a naturally occurring single base change, which disrupts one of the conserved ATG cores and consequently hTEF-5 binding. We further show that PPf/CSEF-1 is immunologically related to the TEA domain and most likely corresponds to a proteolytic product of the TEF factors. Consequently, all of the factors identified to date interacting with the TEF-binding sites in the hCS-B enhancer belong to the TEA domain family. Together, these observations suggest that hTEF-5 is an important regulatory factor in the human placenta.

MATERIALS AND METHODS

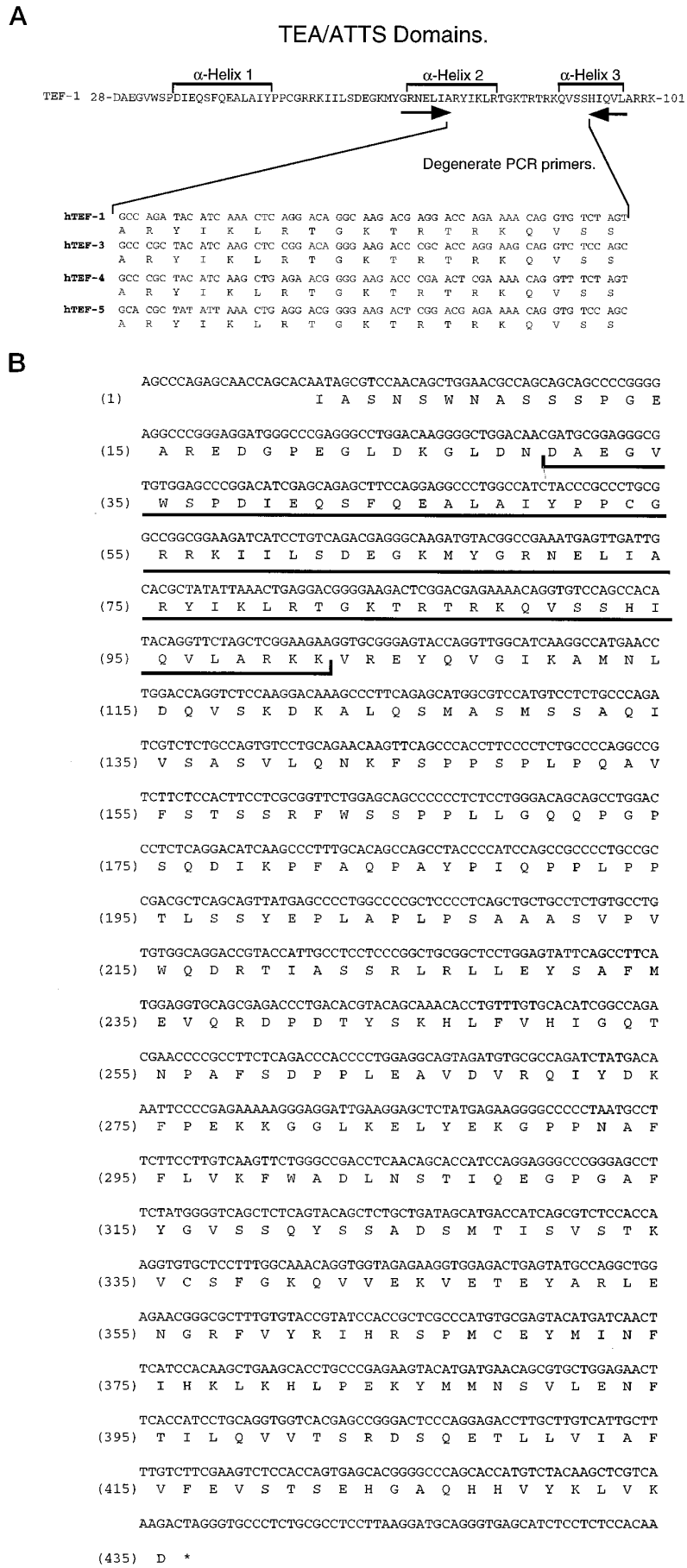
Polymerase Chain Reaction Amplification and Screening of the Placental cDNA Library—Two degenerate oligonucleotides 5′-CCCAAGCTTGGC(A/C)GGAA(C/T)GA(A/G)(C/T)TGAT(A/C)GC-3′ and 5′-CCCAAGCTTC(A/G/C/T)A(G/A)(A/G/C/T)AC(C/T)TG(T/G/A)AT(G/A)TG-3′), corresponding to the TEA domain amino acid sequences GRNELIA and HIQVL, were used as polymerase chain reaction (PCR) primers with a cDNA library of human placental tissue as template. 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, DNA from $>10^6$ plaque-forming units of phage, 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 the placental cDNA library were generated by PCR using the degenerate primers described above and the partial hTEF-5 or full-length hTEF-1, hTEF-3, and hTEF-4 cDNAs as templates in the presence of [α-^{32}P]dCTP. The cDNA library was screened by hybridization at 42 °C in 6 × SSC, 50% formamide. Filters were washed at 55 °C in 3 × SSC. Positive clones were picked and purified, and the cDNA was excised from λ ExLox (Novagen) 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 and Reporter Plasmids—The hTEF-5 open reading frame was amplified with appropriately positioned oligonucleotides containing a consensus Kozak sequence replacing the translation initiation isoleucine codon with ATG. The primers contained *Eco*RI or *Xho*I restriction sites, and the PCR fragment was cloned between the corresponding sites in pXJ41 (1). The DNA sequence of the expression vector was verified by automated DNA sequencing. The expression vectors for the other human and mouse TEFs were as described previously (28). Human and mouse TEF-3A cDNA clones encoding the alternatively spliced isoforms were PCR-amplified with primers containing *Eco*RI/*Xho*I restriction sites as described above and cloned into pXJ41. The hCS-B DF-3 reporter constructs were constructed by PCR using oligonucleotides bearing the appropriate mutations. The resulting fragments were cloned upstream of the thymidine kinase promoter as described previously (43).

Transfections, Preparation of Cell Extracts, and Cloramphenicol Acetyltransferase (CAT) Assays—For EMSA, COS cells were transfected by the calcium phosphate coprecipitation technique as described previously (26, 28). 48 h after transfection, the cells were harvested (from 60-mm diameter dishes), and extracts prepared by three cycles of freeze-thaw in 100 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 of leupeptin, pepstatin, aprotinin, antipain, and chymostatin as described (28, 49). Generally, between 1 and 5 μl of the extracts were then used in EMSA. For CAT assays, 2 μg of reporter constructs and 1 μg of the Rous sarcoma virus-luciferase vector as an internal standard were introduced into JEG-3 cells by lipofection. 48 h after transfection, cell extracts were prepared and luciferase values were determined. After correction for the luciferase values, CAT assays were performed and quantitated on a Fujix BAS 2000 apparatus as described (50). JEG-3 whole cell extracts were prepared as described previously (46, 47).

Electrophoretic Mobility Shift Assays—The oligonucleotides containing the wild-type or mutated GT-IIC enhanson and the tandemly repeated GT-IIC or Sph enhansons were as described previously (1, 26). The oligonucleotides were ^{32}P-5′-end-labeled using polynucleotide kinase and separated from unincorporated [γ-^{32}P]ATP by chromatography on G50-Sepharose. The hCS-A and hCS-B DF-3 and DF-4 fragments were generated by PCR using ^{32}P-5′-end-labeled oligonucleotides 7 and 8 or 2 and 5, respectively, as described (Ref. 43, and see boundaries (*arrows*) in Fig. 6*B*) using the appropriate DNA templates. EMSAs were performed essentially as described previously (1, 26, 28) on 5% polyacrylamide gels in 0.5 × standard TBE buffer. Where indicated, 1–2 μg of the monoclonal antibodies were preincubated with the extracts for 30 min at 25 °C prior to the addition of the oligonucleotides. Immunodepletions were performed by incubating 50 μl of the JEG-3 whole cell extract with 3 μg of the antibodies for 1 h at 4 °C. 50 μl of protein G-Sepharose was then added to the mixture, and incubation was continued for a further hour. The extract was then centrifuged, and aliquots of the supernatant were used for EMSA.

Northern Blot and Reverse Transcription-PCR (RT-PCR)—A Northern blot containing immobilized total RNA from human tissues (CLONTECH) was hybridized with a continuously labeled full-length hTEF-5 probe generated by PCR in the presence of [α-^{32}P]dCTP. Hybridization was performed overnight at 42 °C in buffer containing 6 × SSC and 50%

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      AGCCCAGAGCAACCAGCACAATAGCGTCCAACAGCTGGAACGCCAGCAGCAGCCCCGGGG
(1)                        I  A  S  N  S  W  N  A  S  S  S  P  G  E

      AGGCCCGGGAGGATGGGCCCGAGGGCCTGGACAAGGGGCTGGACAACGATGCGGAGGGCG
(15)   A  R  E  D  G  P  E  G  L  D  K  G  L  D  N  D  A  E  G  V

      TGTGGAGCCCGGACATCGAGCAGAGCTTCCAGGAGGCCCTGGCCATCTACCCGCCCTGCG
(35)   W  S  P  D  I  E  Q  S  F  Q  E  A  L  A  I  Y  P  P  C  G

      GCCGGCGGAAGATCATCCTGTCAGACGAGGGCAAGATGTACGGCCGAAATGAGTTGATTG
(55)   R  R  K  I  I  L  S  D  E  G  K  M  Y  G  R  N  E  L  I  A

      CACGCTATATTAAACTGAGGACGGGGAAGACTCGGACGAGAAAACAGGTGTCCAGCCACA
(75)   R  Y  I  K  L  R  T  G  K  T  R  T  R  K  Q  V  S  S  H  I

      TACAGGTTCTAGCTCGGAAGAAGGTGCGGGAGTACCAGGTTGGCATCAAGGCCATGAACC
(95)   Q  V  L  A  R  K  K  V  R  E  Y  Q  V  G  I  K  A  M  N  L

      TGGACCAGGTCTCCAAGGACAAAGCCCTTCAGAGCATGGCGTCCATGTCCTCTGCCCAGA
(115)  D  Q  V  S  K  D  K  A  L  Q  S  M  A  S  M  S  S  A  Q  I

      TCGTCTCTGCCAGTGTCCTGCAGAACAAGTTCAGCCCACCTTCCCCTCTGCCCCAGGCCG
(135)  V  S  A  S  V  L  Q  N  K  F  S  P  P  S  P  L  P  Q  A  V

      TCTTCTCCACTTCCTCGCGGTTCTGGAGCAGCCCCCCTCTCCTGGGACAGCAGCCTGGAC
(155)  F  S  T  S  S  R  F  W  S  S  P  P  L  L  G  Q  Q  P  G  P

      CCTCTCAGGACATCAAGCCCTTTGCACAGCCAGCCTACCCCATCCAGCCGCCCCTGCCGC
(175)  S  Q  D  I  K  P  F  A  Q  P  A  Y  P  I  Q  P  P  L  P  P

      CGACGCTCAGCAGTTATGAGCCCCTGGCCCCGCTCCCCTCAGCTGCTGCCTCTGTGCCTG
(195)  T  L  S  S  Y  E  P  L  A  P  L  P  S  A  A  A  S  V  P  V

      TGTGGCAGGACCGTACCATTGCCTCCTCCCGGCTGCGGCTCCTGGAGTATTCAGCCTTCA
(215)  W  Q  D  R  T  I  A  S  S  R  L  R  L  L  E  Y  S  A  F  M

      TGGAGGTGCAGCGAGACCCTGACACGTACAGCAAACACCTGTTTGTGCACATCGGCCAGA
(235)  E  V  Q  R  D  P  D  T  Y  S  K  H  L  F  V  H  I  G  Q  T

      CGAACCCCGCCTTCTCAGACCCACCCCTGGAGGCAGTAGATGTGCGCCAGATCTATGACA
(255)  N  P  A  F  S  D  P  P  L  E  A  V  D  V  R  Q  I  Y  D  K

      AATTCCCCGAGAAAAAGGGAGGATTGAAGGAGCTCTATGAGAAGGGGCCCCCTAATGCCT
(275)  F  P  E  K  K  G  G  L  K  E  L  Y  E  K  G  P  P  N  A  F

      TCTTCCTTGTCAAGTTCTGGGCCGACCTCAACAGCACCATCCAGGAGGGCCCGGGAGCCT
(295)  F  L  V  K  F  W  A  D  L  N  S  T  I  Q  E  G  P  G  A  F

      TCTATGGGGTCAGCTCTCAGTACAGCTCTGCTGATAGCATGACCATCAGCGTCTCCACCA
(315)  Y  G  V  S  S  Q  Y  S  S  A  D  S  M  T  I  S  V  S  T  K

      AGGTGTGCTCCTTTGGCAAACAGGTGGTAGAGAAGGTGGAGACTGAGTATGCCAGGCTGG
(335)  V  C  S  F  G  K  Q  V  V  E  K  V  E  T  E  Y  A  R  L  E

      AGAACGGGCGCTTTGTGTACCGTATCCACCGCTCGCCCATGTGCGAGTACATGATCAACT
(355)  N  G  R  F  V  Y  R  I  H  R  S  P  M  C  E  Y  M  I  N  F

      TCATCCACAAGCTGAAGCACCTGCCCGAGAAGTACATGATGAACAGCGTGCTGGAGAACT
(375)  I  H  K  L  K  H  L  P  E  K  Y  M  M  N  S  V  L  E  N  F

      TCACCATCCTGCAGGTGGTCACGAGCCGGGACTCCCAGGAGACCTTGCTTGTCATTGCTT
(395)  T  I  L  Q  V  V  T  S  R  D  S  Q  E  T  L  L  V  I  A  F

      TTGTCTTCGAAGTCTCCACCAGTGAGCACGGGGCCCAGCACCATGTCTACAAGCTCGTCA
(415)  V  F  E  V  S  T  S  E  H  G  A  Q  H  H  V  Y  K  L  V  K

      AAGACTAGGGTGCCCTCTGCGCCTCCTTAAGGATGCAGGGTGAGCATCTCCTCTCCACAA

(435)  D  *
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FIG. 1. *A*, sequences of human TEA domains. The *upper part* shows the amino acid sequence of human TEF-1. 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 TEA domains shown in the *lower part*. The *lower part* shows the nucleotide and amino acid sequences of the PCR-amplified TEA domain subregions from hTEF-1, hTEF-3, hTEF-4, and hTEF-5. *B*, the complete nucleotide and amino acid sequence of hTEF-5 is shown. The amino acid coordinates are indicated by the *numbers* in *parentheses*. The location of the TEA domain is indicated.

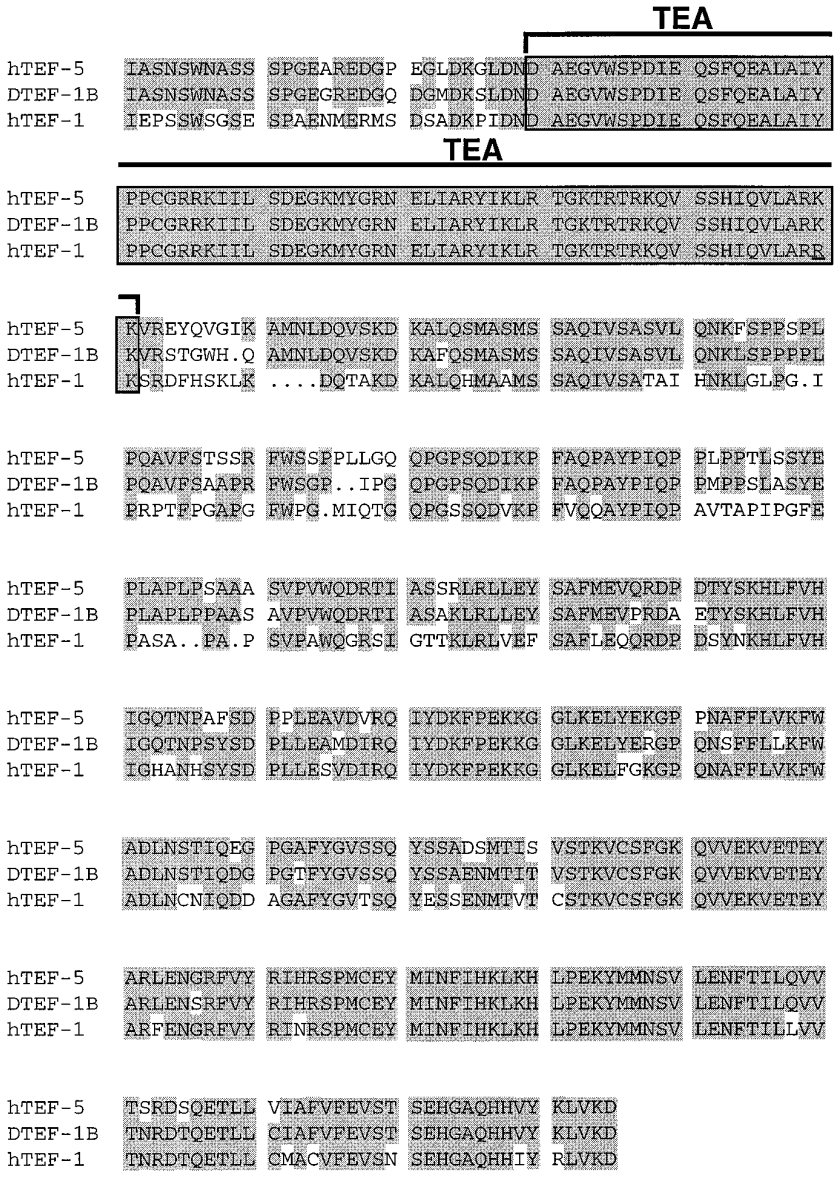

FIG. 2. **Alignment of the amino acid sequences of hTEF-5, chicken DTEF-1B, and hTEF-1.** Amino acids conserved in at least two of the three proteins are *boxed*. The position of the TEA domain is indicated, and the R100K amino acid substitution is *underlined*.

formamide. The blot was washed with 0.3 × SSC at 50 °C and subjected to autoradiography. After exposure, the blot was stripped and hybridized to a probe for cytoskeletal β-actin to verify that each lane contained RNA. For RT-PCR, total cytoplasmic RNA was isolated from the 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 common to all three RNA polymerases, generating a 630-nucleotide fragment (Ref. 51 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-4, 5′-CTTGGACTG-GATTTCCCT-3; and hTEF-5, 5′-ACCTGGTACTCCCGCACC-3′. The products of reverse transcription were then amplified using the same antisense primers and the following sense primers: hTEF-4, 5′-GGGGGTGACGGGGGCCCG-3′; and hTEF-5, 5′-AACGCCAGCAG-CAGCCCC-3′. The 5′ and 3′ primers were chosen in separate exons to distinguish the cDNA product from possible contaminating genomic DNA. RT-PCR generated a 255-nucleotide fragment for hTEF-4 and a 302-nucleotide fragment for hTEF-5. Control PCR reactions were performed using 10 pg of the appropriate expression vectors or no DNA template. 30 cycles of PCR were performed with 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*A*.

Expression of the TEF-1 TEA Domain and Monoclonal Antibody Production—The region of TEF-1 encoding the TEA domain (amino acids 28–104) was PCR-amplified with primers containing *Bam*HI and *Eco*RI restriction sites, and the PCR product was cloned into the vector pGEX2T. The plasmid was transformed into the *Escherichia coli* DH5 strain, and expression of the fusion protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside for 2 h. The fusion protein was purified by chromatography on glutathione-Sepharose (Pharmacia Biotech Inc.), eluted with reduced glutathione, and analyzed by SDS-polyacrylamide gel electrophoresis. Immunizations and monoclonal antibody production were performed as described previously (52–54). Briefly, mice were injected intraperitoneally three times at 2-week intervals with the purified GST-TEA fusion protein. Spleen cells were fused with Sp2/O AG 14 myeloma cells, and culture supernatants at day 10 were tested on COS cells transfected with pXJ40-TEF-1 by immunofluorescence or by enzyme-linked immunosorbent assay. The antibodies were also characterized by Western blotting against the GST-TEA domain fusion protein and the 6-His-tagged TEA domain protein (28). After generation of ascites fluid, the antibodies were purified by caprylic acid and ammonium sulfate precipitation as described previously (52, 53). mAbs 3G3, 22TA, and 2GV3 are as described previously (52–56).

RESULTS

Isolation of a cDNA Encoding hTEF-5, a Homologue of Chicken DTEF-1—To isolate TEF factors expressed in human

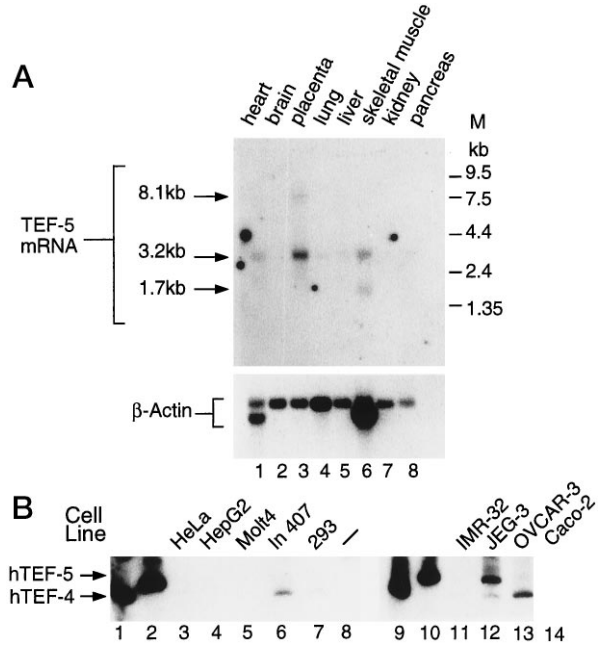

FIG. 3. *A*, Northern blot analysis of hTEF-5 expression. A Northern blot (CLONTECH) containing human RNA from the tissues indicated *above* each *lane* was hybridized with a ^{32}P-labeled hTEF-5 DNA probe. The positions of the molecular mass standards are indicated to the *right*, and the hTEF-5 mRNAs are indicated to the *left*. The *lower part* shows a rehybridization of the same filter with the β-actin probe. *B*, expression of TEF-4 and hTEF-5 in human cell lines. RT-PCR was used to amplify fragments of the TEF-4 and hTEF-5 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*, *2*, *9*, and *10* show the amplification product generated using 10 pg of the corresponding expression vectors as templates, and *lane 8* shows the product generated with no 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 an ovary adenocarcinoma, JEG-3 from a choriocarcinoma, and CaCo-2 from a colon adenocarcinoma. Intestine (*In*) 407 cells are from embryonic intestine, and 293 cells are transformed embryonal kidney cells.

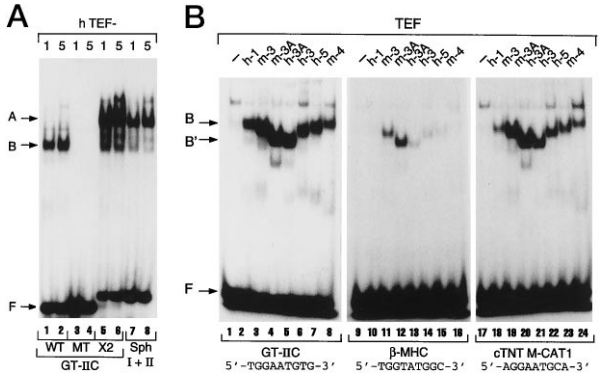

FIG. 4. *A*, binding of TEF-1 and hTEF-5 to the GT-IIC and Sph enhansons from the SV40 enhancer. EMSA was performed with the ^{32}P-labeled oligonucleotides used as indicated *below* each *lane* with extracts from COS cells transfected with the expression vectors for the TEF factors shown *above* each *lane*. *WT*, wild type; *MT*, mutant. ×2 indicates that the sites are organized as a tandem repeat. The specific complexes B and A, in which one or both sites are occupied, are indicated along with the free probe (*F*). *B*, comparison of TEF binding to sites in muscle-specific promoters. The transfected TEFs used are shown *above* each *lane*. The sequences of the TEF binding sites in the oligonucleotide probes are shown at the *bottom* of each *panel*. *Lanes 1–8*, the GT-IIC enhanson of the SV40 enhancer; *lanes 9–16*, the β-myosin heavy chain (*β-MHC*) gene promoter proximal enhancer core element from −200 to −215 (37); *lanes 17–24*, the cardiac troponin T (*cTNT*) M-CAT1 motif from −83 to −101 (11). The specific B complex is indicated along with the analogous complex (*B′*) generated with the TEF-3A isoforms. *F*, free probe.

placenta, degenerate oligonucleotides corresponding to the conserved amino acids GRNELIA and the complement of HIQVL in α-helices 2 and 3 of the TEA domain (see Fig. 1*A*) were used for PCR amplification of a human placental cDNA library (see "Materials and Methods"). PCR products of the expected size were cloned, and their DNA sequences were determined. All of the TEA domain sequences analyzed encoded hTEF-5, which contained a TEA domain of identical amino acid sequence to that of hTEF-1 but with a different codon usage (Fig. 1*A*). The partial cDNA was used to isolate a full-length hTEF-5 cDNA. Full-length hTEF-5 initiates at an ATA codon and comprises 435 amino acids (Fig. 1*B*). The TEA domain is identical to that of hTEF-1 except for a conservative Arg to Lys change at amino acid 100. Comparison of the sequence of hTEF-5 with that of the other TEF factors cloned to date showed that hTEF-5 is most closely related to the B isoform of chicken DTEF-1 (93% homology compared with 84% for hTEF-1, 83% for hTEF-3, and 79% for hTEF-4; see also Fig. 2). As previously noted for the other TEFs, the TEA domain and C-terminal regions are best conserved, while the N-terminal region and the region following the TEA domain are the most variable.

To determine whether hTEF-5 was the only TEF expressed in placenta, the cDNA library was also screened at low stringency with a mixture of probes for hTEF-1, hTEF-3, and hTEF-5, and 32 clones were analyzed. Of these, 30 encoded hTEF-5, while two encoded TEF-1. Remarkably, these two independent clones encoded splice variants of TEF-1, where the sequence GKTRTRKQ in the TEA domain is followed by an unrelated sequence comprising VK and a stop codon resulting in a truncated protein, which cannot bind DNA. In addition to the full-length hTEF-5, one clone encoding an alternatively spliced isoform of hTEF-5 was isolated. This isoform contained the sequence GKTRTRKQ of the TEA domain fused directly to a downstream exon beginning AMNLDQSV. The protein encoded by this splice variant lacks α-helix three of the TEA domain and consequently does not bind DNA (data not shown). These results show that hTEF-5 is the only full-length TEF protein encoded in the placental cDNA library.

hTEF-5 Is Preferentially Expressed in Skeletal Muscle and Placenta—The expression pattern of hTEF-5 in adult human tissues was investigated by Northern blot analysis. Strong expression of a 3.2-kilobase mRNA was detected in placenta and skeletal muscle and more weakly in heart (Fig. 3*A*, *lanes 3*, *6*, and *1*, respectively). In addition to the 3.2-kilobase mRNA, an 8.1-kilobase mRNA was detected only in placenta, while a shorter 1.7-kilobase transcript was detected only in skeletal muscle.

We next compared the expression patterns of hTEF-5 and hTEF-4, which also has a restricted expression pattern (28), in cultured cell lines. Exon-specific oligonucleotides in hTEF-4 and hTEF-5 were used in an RT-PCR assay (see "Materials and Methods"). As described previously, hTEF-4 transcripts were readily detected in intestine (*In*) 407 and ovarian OVCAR-3 cells and weakly in 293 and JEG-3 cells (Fig. 3*B*, *lanes 6*, *13*, *7*, and *12*, respectively). In contrast, hTEF-5 was expressed only in JEG-3 choriocarcinoma cells (Fig. 3*B*, *lane 12*). Together with the results of the Northern blot analysis, this indicates that hTEF-5 is strongly expressed in placenta and placenta-derived cells.

hTEF-5 Binds to TEF-binding Sites in the SV40 Enhancer and in Muscle Promoters—The hTEF-5 coding sequence was inserted into the pXJ41 (1) eukaryotic expression vector and transfected into COS cells. The transfected cell extracts were then used in EMSAs. Both TEF-1 and hTEF-5 bound specifi-

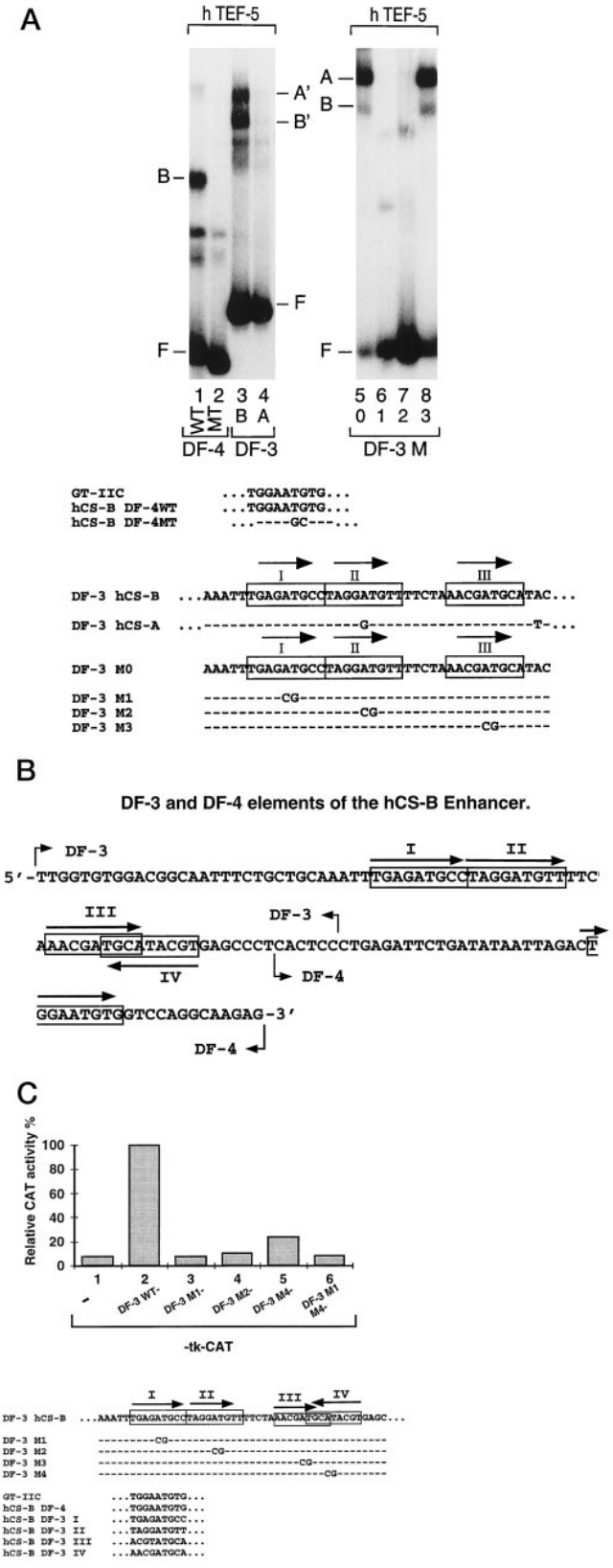

FIG. 5. *A*, binding of hTEF-5 to the hCS-B DF-4 and hCS-A and hCS-B DF-3 elements. EMSA was performed with PCR fragments containing the hCS-B DF-4 element (*lanes 1* and *2*) or the DF-3 element from the hCS-B or A enhancers (*lanes 3* and *4*). The sequences of the TEF-binding sites in each enhancer are indicated at the *bottom* of the *panel*. In *lanes 5–8*, EMSA was performed with the wild-type or mutated oligonucleotides whose sequences are indicated at the *bottom* of the *panel*. The positions of the specific complexes A and B are indicated along with that of the free probe (*F*). *B*, the sequence of the human hCS-B enhancer. The boundaries of the DF-3 and DF-4 elements are indicated by *arrows*. The potential hTEF-5 binding sites I–IV in DF-3 and the binding site in DF-4 are *boxed*. *C*, functional analysis of the hCS-B DF-3 hTEF-5 binding sites. The *graph* shows a quantitative PhosphorImager analysis of CAT assays performed after lipofection of the constructs shown into placental JEG-3 cells. The activity of the wild-type DF-3 element has been taken as 100%. The sequences of the DF-3 hTEF-5 binding sites in each construct are shown *below* the *graph*. An alignment of the sequences of the hCS-B and GT-IIC sites is also shown *below* the *graph*.

cally to the GT-IIC enhanson of the SV40 enhancer (Fig. 4*A*, *lanes 1–4*). Both proteins also bound cooperatively to the tandemly repeated GT-IIC and Sph enhansons as evidenced by the preferential formation of complex A, where both enhansons are occupied (Fig. 4*A*, *lanes 5–8*).

The relative binding efficiencies of hTEF-5 and the other TEF factors to sites present in several muscle-specific promoters were investigated. As described previously, hTEF-1, hTEF-3, hTEF-5, mTEF-3, and mTEF-4 all bound to the GT-IIC enhanson (Fig. 4*B*, *lanes 2*, *6*, *7*, *3*, and *8*, respectively). Similarly, binding of an alternatively spliced isoform of mouse and hTEF-3 lacking the exon after the TEA domain (TEF-3A; Refs. 31 and 32), was observed (Fig. 4*B*, *lanes 4* and *5*). Using the same amounts of transfected cell extracts that gave equivalent binding to the GT-IIC site, similar relative binding efficiencies to the M-CAT1 motif of the cardiac troponin T (*cTNT*) promoter were observed (Fig. 4*B*, *lanes 17–24*; Ref. 11). However, all of these factors bound with lower affinity to the proximal enhancer core motif of the rat β-myosin heavy chain (*MHC*) gene promoter (Fig 4*B*, *lanes 9–16*; Ref. 37), whose sequence differs from the consensus (5′-ATG GC-3′; consensus 5′-ATG(C/T)(G/A)-3′). Quantitative PhosphorImager analysis indicated that the binding of hTEF-1 and mTEF-4 to this site was at least 2–3-fold weaker than that of mTEF-3, hTEF-3, and hTEF-5. These results show that all of the TEF factors bind to the TEF-binding sites in several muscle-specific promoters, albeit with slightly different affinities to the β-myosin heavy chain site.

hTEF-5 Binds to a Novel Functional Element of the hCS-B Enhancer—We next examined the binding of hTEF-5 to the enhansons of the placenta-specific hCS-B enhancer. Radiolabeled probes containing the DF-3 and DF-4 elements (Ref. 43; see Fig. 5*B*) were generated by PCR and used in EMSA. hTEF-5 bound specifically to the consensus TEF-binding site in the wild-type DF-4 (Refs. 42, 43, and 45; see Fig. 5, *A* and *B*) but not to a DF-4 in which this site had been mutated (Fig. 5*A*, *lanes 1* and *2*). This same mutation inactivates the DF-4 element in JEG-3 cells and syncytiotrophoblast (46, 47).

A comparison of the activities of the closely related hCS-A and hCS-B DF-3 elements has shown that the hCS-A element is inactive in JEG-3 cells and syncytiotrophoblast (46, 47). These DF-3 elements differ by two nucleotide changes (Fig. 5*A*). In EMSA, hTEF-5 binds to the hCS-B DF-3 fragment to generate two complexes, A′ and B′, which are not formed on the equivalent fragment from the hCS-A enhancer (Fig. 5*A*, *lanes 3* and *4*). This observation suggests that one or both of the nucleotide changes disrupt an hTEF-5 binding site(s). Several potential TEF binding sites have already been pointed out in this region (Ref. 45; Fig. 5, *A* and *B*, *sites III* and *IV*); however, our inspection of the DF-3 sequence revealed two additional potential TEF-binding sites containing an ATG core (Fig. 5, *A* and *B*, *sites I* and *II*) arranged as a tandem repeat analogous to the Sph motifs in the SV40 enhancer. One of the base changes in the hCS-A enhancer mutates the ATG core of site II (Fig. 5*A*). To investigate the binding of hTEF-5 to these sites, oligonucleotides containing wild-type or mutated versions of sites

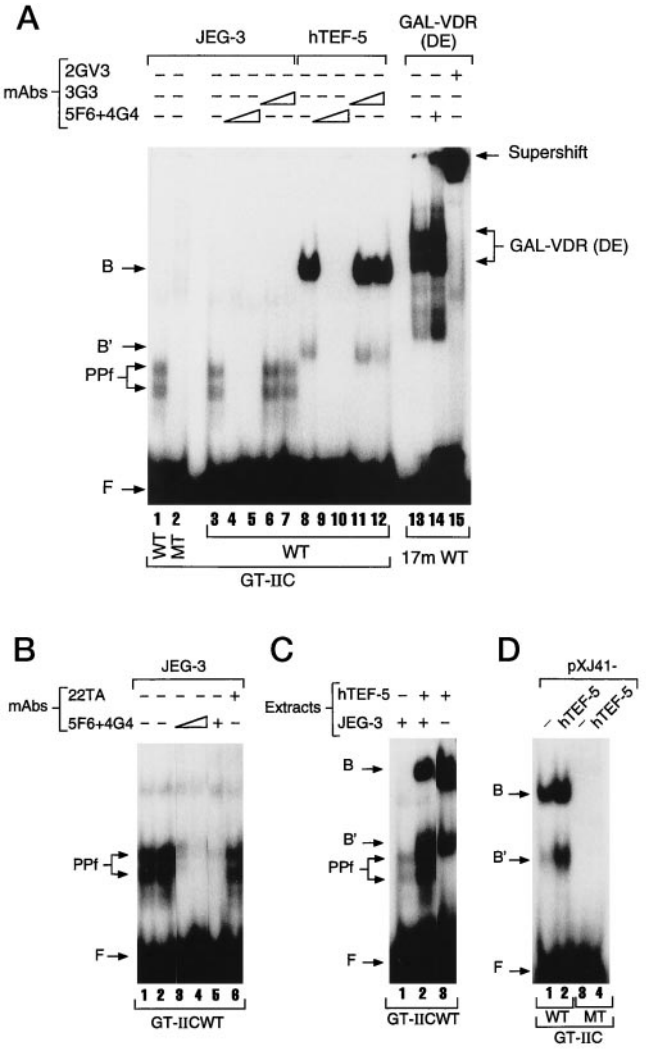

FIG. 6. *A*, PPf/CSEF-1 is immunologically related to the TEF factors. The oligonucleotides used are indicated at the *bottom* of the *panel*, and the cell extracts are shown at the *top*. *GT-IIC WT* and *MT* indicate the wild-type and mutated probes, respectively. 17m WT contains a single 17-mer GAL4 binding site. The presence of the mAbs added to the reactions is indicated *above* each *lane*. *Lane 14* contains the same concentration of mAbs 5F6 and 4G4 as *lanes 5* and *10*. *PPf* indicates the complexes formed by the JEG-3 cell PPf factor, *B* shows the complex formed by the full-length hTEF-5, and *B′* represents the complex formed by the proteolytic degradation product present in the transfected cell extracts. *Supershift* indicates the GAL-VDR(DE)-DNA complex supershifted by mAb 2GV3. *B*, the layout is as in *A*. In *lanes 3* and *4* the mAbs were added to the EMSA reaction, whereas *lanes 5* and *6* show the extracts immunodepleted with the mAbs indicated *above* each *lane*. *C*, *lanes 1* and *3* show the JEG-3 cell extract alone and the hTEF-5-transfected COS cell extract alone incubated at 37 °C for 30 min prior to EMSA. In *lane 2*, the cell extracts were mixed together and incubated as described above. *B* indicates the complex formed by the binding of full-length TEF-5, and *B′* represents the complex formed by a proteolytic digestion product. *D*, freeze-thaw rather than whole cell extracts were prepared from JEG-3 cells transfected with the expression vectors shown *above* each *lane*. *B* and *B′* are as indicated in *C*.

I–IV were used in EMSA. The binding of hTEF-5 to the wild-type oligonucleotide generated two retarded complexes, *A* and *B* (Fig. 5*A*, *lane 5*), analogous to those formed with the Sph enhansons, where complex A, in which both binding sites are occupied, is preferentially formed. Formation of these complexes was unaffected by mutation of site III (*lane 8*), whereas formation of both complexes was abolished by mutations in site I or II (*lanes 6* and *7*). These results show that hTEF-5 binds cooperatively to the tandemly repeated sites I and II but not to site III or IV (data not shown). These results define novel tandemly repeated TEF-binding sites within the DF-3 element of the hCS-B enhancer.

To address the functional importance of these sites, a wild-type DF-3 element or DF-3 elements mutated in sites I, II, and IV were inserted upstream of the thymidine kinase promoter driving expression of the bacterial CAT gene. These reporter constructs were transfected into JEG-3 cells, and the resulting CAT activity was quantified by PhosphorImager analysis. The wild-type DF-3 element strongly stimulated CAT activity compared with the native thymidine kinase promoter (Fig. 5*C*, *bars 1* and *2*). The mutations in sites I and II that disrupted hTEF-5 binding resulted in an almost complete loss of DF-3 activity (*bars 3* and *4*). Mutation of site IV also resulted in a significant loss of activity (*bar 5*), while simultaneous mutation of sites I and IV completely abolished activity (*bar 6*). These results indicate that the hTEF-5 binding sites I and II are critical for the activity of the DF-3 element. Although site IV does not bind hTEF-5, the factor(s) that binds to this site cooperates with the TEF-binding sites I and II to generate DF-3 activity.

PPf/CSEF-1 Is Immunologically Related to the TEF Factors—The above results indicate that hTEF-5 is expressed in placenta and binds to several functional enhansons in the hCS-B enhancer. However, it has previously been reported that the DF-4 TEF-binding site is recognized by the low molecular mass PPf/CSEF-1, which in EMSA generates a complex with a different electrophoretic mobility from that of hTEF-5 (43, 46–48; see Fig. 6*A*). PPf recognizes the same sequence as hTEF-5, suggesting that this protein is either a TEF isoform or degradation product or an unrelated protein with the same binding specificity. Although it has been reported that CSEF-1/PPf is not related to the TEFs (48), we reasoned that due to its reported low molecular mass it may comprise little more than the TEA domain and the immediately surrounding sequences. To test this possibility, we generated monoclonal antibodies to the TEA domain (mAbs 4G4 and 5F6; see "Materials and Methods") and used these antibodies in EMSA.

Incubation of oligonucleotides comprising the GT-IIC enhanson with JEG-3 whole cell extracts (WCE) generated specific PPf complexes (Fig. 6, *A* and *B*, *lanes 1* and *2*; Ref. 43) of much higher electrophoretic mobility than those observed with extracts of cells transfected with hTEF-5 (Fig. 6*A*, *lane 8*). Preincubation of the JEG-3 WCE or transfected COS cell extracts with mAbs 4G4 and 5F6 inhibited binding of hTEF-5 (Fig. 6*A*, *lanes 9* and *10*) and the formation of the PPf complexes (Fig. 6*A*, *lanes 4* and *5*). In contrast, control mAbs against the TATA-binding protein (mAb 3G3 (52–54)) had no effect on the binding

of hTEF-5 or PPf (Fig. 6*A*, *lanes 6* and *7* and *lanes 11* and *12*). Similarly, the anti-TEA domain antibodies did not inhibit the binding of a fusion protein containing the GAL4 DBD (in this case GAL-VDR(DE) (50)) to oligonucleotides containing a GAL4 binding site (Fig. 6*A*, *lanes 13* and *14*), while the GAL-VDR(DE)-DNA complexes were supershifted by the anti-GAL4 DBD mAb 2GV3 (Ref. 56; Fig. 6*A*, *lane 15*).

In an alternative assay, the JEG-3 WCE was immunodepleted with mAbs 4G4 and 5F6 or with an mAb directed against human TATA-binding protein-associated factor 20 (mAb 22TA; Ref. 55), and aliquots of the immunodepleted extracts were used in EMSA. Using JEG-3 WCE immunodepleted with mAb 4G4 or 5F6, no PPf complexes were formed (Fig. 6*B*, *lane 5*), whereas these complexes were formed in extracts immunodepleted with the control mAb 22TA (Fig. 6*B*, *lane 6*). These results demonstrate that the PPf/CSEF-1 protein(s) are immunologically related to the TEA domain.

One explanation for the above results is that PPf is a proteolytic breakdown product of hTEF-5 or another TEF generated by a protease present in the JEG-3 WCE. To test this possibility, we incubated the transfected COS cell extracts either alone or with the JEG-3 WCE for 30 min at 37 °C prior to EMSA. When incubated alone, only minor proteolytic degradation was observed with the hTEF-5-transfected COS cell extract, and the resulting faster migrating complex (*B′*) had an electrophoretic mobility different from that of the PPf complexes (Fig. 6*C*, *lane 3*). In contrast, when incubated with an aliquot of the JEG-3 WCE the amount of complex B generated by the binding of full-length hTEF-5 was significantly decreased with a concomitant increase in the PPf complexes (Fig. 6*C*, *lanes 1* and *2*). Thus, a protease present in JEG-3 WCE, but not in transfected COS cell extract, degrades hTEF-5, generating proteolytic fragments that form the PPf complexes.

We next determined whether the PPf breakdown products were the predominant TEF species present in JEG-3 cells or whether they were artefactually formed during the extraction procedure. To answer this question, extracts from JEG-3 cells transfected with pXJ41 or pXJ41-hTEF-5 were prepared, not by the WCE procedure, but by the simpler freeze-thaw protocol, and EMSA was performed. When extracts were prepared in this way from the hTEF-5-transfected cells, the predominant specific complex (*B*) was formed by the binding of full-length hTEF-5 (Fig. 6*D*, *lanes 2* and *4*). Strikingly, this complex was also the predominant specific complex formed in extracts from mock-transfected cells, while no PPf complexes were observed (*lanes 1* and *3*). Thus, the extracts made using the freeze thaw procedure, either from JEG-3 or COS cells, do not contain an active form of the protease that cleaves TEF-5 into the PPf product, although the B′ product is still observed. These two proteolytic products are therefore likely to be generated by distinct proteases. These results further show that the TEF factors in JEG-3 cells are mainly full-length.

DISCUSSION

TEF-5 Is a Mammalian Homologue of Chicken DTEF-1—We report here the cloning of hTEF-5, related to the previously described human and murine TEF-1, -3, and -4 factors. Analysis of the amino acid sequence of hTEF-5 shows that, similar to TEF-1 and TEF-3 (1, 28), the protein initiates with a non-ATG codon, in this case ATA. The TEA domain of hTEF-5 contains only one amino acid substitution compared with that of TEF-1, and, as observed with the other TEFs, the C-terminal region is also highly conserved, but the N-terminal region and the region following the TEA domain are more divergent (28).

Comparison of the hTEF-5 amino acid sequence with other cloned TEFs indicated that hTEF-5 is most closely related to the B isoform of chicken DTEF-1 (84% identity). The TEA domain of the DTEF-1 B isoform contains the R100K amino acid substitution found in hTEF-5; however, none of the hTEF-5 clones analyzed contained the R87K and I94L substitutions found in the DTEF-1 A isoform (33). These results show that the vertebrate genome contains at least four highly related TEF genes conserved from chickens to humans.

The expression patterns of hTEF-5 and DTEF-1 may be somewhat different. In chickens, DTEF-1 is most strongly expressed in embryonic cardiac muscle, while very low levels are observed in skeletal muscle (33). On the other hand, in adults, hTEF-5 is expressed at approximately equivalent levels in both of these tissues. DTEF-1 is also expressed at moderate levels in the lung, whereas only trace levels of expression are detected for hTEF-5. Most strikingly, however, the predominant site of hTEF-5 expression is the placenta, a tissue that has no equivalent in chicken.

The expression of several TEFs is enriched in skeletal and cardiac muscle, and a variety of TEF target genes have been described in these tissues. This report together with previous results from our own and other laboratories (13, 17, 31–37, 57) show that all of the TEF factors can bind with similar relative affinities to sites present in the regulatory regions of several target genes. In adult skeletal muscle, TEF-1, TEF-3, and hTEF-5 are all expressed, suggesting that they may play redundant roles. In keeping with this idea, TEF-1 null mice show defects only in cardiogenesis, although this does not exclude the possibility that each TEF factor plays a specific role in skeletal muscle development and that additional functions of TEF-1 would have become evident only at stages subsequent to that at which the TEF-1 null embryos die. Moreover, we proposed (28) that the phenotype of the TEF-1 null mice reflected the fact that TEF-1 was the only TEF expressed in the developing myocardium. Since our present results show that hTEF-5 is expressed in adult cardiac muscle, further *in situ* hybridizations comparing the expression of TEF-1 and TEF-5 in the developing mouse embryo will be required to determine whether these two genes are coexpressed or are expressed at different stages of cardiogenesis.

A Potential Role for hTEF-5 in Placenta-specific Gene Expression—The predominant site of hTEF-5 expression is the placenta. This observation is consistent with the restricted expression of hTEF-5 in JEG-3 cells. We have previously reported that TEF-1, TEF-3, and TEF-4 were also expressed in JEG-3 cells (28); however, our present results suggest that this is not the case in the placenta. Low stringency screening of a placental cDNA library with probes for each TEF resulted in isolation of many independent hTEF-5 clones, two clones encoding truncated splice variants of hTEF-1, but no clones for hTEF-3 or hTEF-4. Although the splice variants of TEF-1 do not bind DNA, we cannot formally rule out the possibility that they perform some other function. Nevertheless, hTEF-5 is the predominant TEF expressed in the placenta.

To relate the placental expression of hTEF-5 to that of potential target genes, we show that hTEF-5 binds to multiple functional sites in the hCS-B enhancer. The DF-4 site, which is identical to the GT-IIC enhanson, binds hTEF-5, and mutations that inhibit hTEF-5 binding inactivate the DF-4 element. Other potential TEF binding sites have been pointed out in the DF-3 element. Several of these (see, for example, EM_6 in Ref. 45) are unlikely to be bona fide TEF-binding sites, since they lack the conserved ATG core sequence. However, two other sites identified in that study (see EM_4, designated sites III and IV in this study) contain an ATG core, but despite this homology, no binding of hTEF-5 (or TEF-1)[2] was observed.

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

Nevertheless, adjacent to sites III and IV we identified two other binding sites, organized as a tandem repeat reminiscent of the Sph enhansons, to which hTEF-5 binds cooperatively as shown by the preferential formation of complex A. When either of the two sites was mutated, no binding to the remaining site was observed, showing that individually these are low affinity binding sites and that the integrity of both sites and cooperativity is required for efficient binding to this element. In correlation with these observations, mutations in either of the two sites that disrupt hTEF-5 binding also inactivate the DF-3 element. These results clearly define a novel functional element in the hCS-B enhancer comprising tandemly repeated hTEF-5 binding sites.

In contrast to the hCS-B DF-3 element, the hCS-A DF-3 element is inactive in placenta-derived cell lines and syncytiotrophoblast (46, 47). This differential activity correlates with hTEF-5 binding, since the hCS-B DF-3 binds hTEF-5, but the hCS-A DF-3 does not. The lack of hTEF-5 binding can be ascribed to the fact that one of the mutations in the hCS-A DF-3 element affects the ATG core sequence of binding site II. It has been reported that back-mutation of this nucleotide to the hCS-B sequence (*i.e.* G → A) results in a gain of function (58). These authors have shown that sequences in this region bind two unidentified proteins present in JEG-3 extracts. However, in these experiments the oligonucleotides used did not contain the complete nucleotide sequences of sites I and II and, consequently, probably do not bind hTEF-5. Thus, although our results do not exclude the possibility that other proteins bind to this region, we show that the placentally expressed hTEF-5 binds cooperatively to tandemly repeated sites in this region and that this binding correlates with the function of the DF-3 element.

At first sight, a more direct approach to determine the role of hTEF-5 in hCS enhancer function would be to express hTEF-5 with an appropriate reporter construct in HeLa cells that do not express hTEF-5 and where the hCS enhancer is inactive. However, overexpression of hTEF-5 does not activate transcription from the hCS-B enhancer either in HeLa or in JEG-3 cells.[2] These observations may be explained in two ways. First, as previously proposed (44, 58), the hCS enhancer contains negative regulatory sequences, which repress its activity in nonplacental cells; thus, expression of hTEF-5 alone would not be sufficient to activate this enhancer. Second, even in JEG-3 cells, overexpression of hTEF-5 does not further activate, but rather represses, hCS enhancer activity due to a transcriptional interference/squelching effect previously observed with TEF-1 (1, 26). Expression of hTEF-5 in HeLa cells represses the activity of endogenous HeLa cell TEF-1 showing that these two factors compete for a common limiting intermediary factor.[2] To unambiguously determine the role of hTEF-5 in placental gene expression, it will be necessary to inactivate this gene in the mouse and analyze the resulting phenotype. Experiments of this type are currently in progress.

In contrast to previous reports (48), we show here that the PPf/CSEF-1 proteins are immunologically related to the TEF factors, since formation of the PPf complexes is inhibited by mAbs against the TEA domain. It is probable that PPf corresponds to a proteolytic breakdown product of hTEF-5, since incubation of hTEF-5 with JEG-3 WCE results in the disappearance of the full-length hTEF-5 and the appearance of PPf complexes. The resulting proteolytic fragment is considerably smaller than the full-length protein, explaining the previously reported differences in chromatographic properties and thermal stability between PPf/CSEF-1 and TEF-1 (48). The lack of cross-reaction with anti-cTEF-3 antibodies may be explained if the corresponding epitope is either not conserved between cTEF-3 and hTEF-5 and/or if the epitope is not present in the proteolytic fragment. Together these results show that the previously described PPf/CSEF-1 factor is in fact a selective proteolytic degradation product of the TEF factors.

Previous studies have highlighted the correlation between hCS-B enhancer activity and the presence of the PPf proteolysis products, raising the possibility that proteolysis of the TEF factors may be important for their function. However, this idea is not supported by the observation that, when an alternative extraction procedure is used, predominantly full-length TEF factors are detected, showing that an artefactual, but selective proteolysis occurs during extract preparation. In conclusion, our results demonstrate that all of the factors described to date that interact with the TEF-binding elements of the hCS-B enhancer belong to the TEF family of transcription factors, further highlighting the potential role of these factors in placental transcription.

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