Novel Human TEF-1 Isoforms Exhibit Altered DNA Binding and Functional Properties[†]

Shi-Wen Jiang,[‡] Miguel A. Trujillo,[‡] Michiko Sakagashira,[‡] Russell A. Wilke,[‡] and Norman L. Eberhardt*[,‡,§]

Endocrine Research Unit, Departments of Medicine and Biochemistry/Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

Received May 7, 1999; Revised Manuscript Received October 11, 1999

ABSTRACT: The transcriptional enhancer factor-1 (TEF-1) is a member of the TEA/ATTS domain family. TEF-1 binds to GT-IIC (GGAATG), SphI (AGTATG), SphII (AGCATG), and M-CAT (GGTATG) response elements and is involved in the transactivation of a variety of genes, including the SV40 large T antigen, mammalian muscle-specific genes, and human chorionic somatomammotropin genes. Also, TEF-1 acts as a transcriptional repressor in placental cells, possibly through interaction with the TATA binding protein (TBP), preventing TBP binding to the TATA box. Here we describe the cloning, tissue-specific expression pattern, and functional characterization of two novel TEF-1 isoforms, TEF-1β and TEF-1γ. These isoforms most likely arise from alternative splicing of mRNA transcribed from a single gene and involve substitutions and/or insertions in a region immediately following the DNA binding domain. TEF-1β appears to be widely distributed like the prototypic TEF-1, designated TEF-1α, whereas TEF-1γ exhibits a narrower tissue-specific expression pattern that includes pancreas, kidney, and skeletal and heart muscle. The relatively limited sequence alterations among these isoforms cause significant changes in their DNA binding and transcriptional activities. TEF-1β and TEF-1γ bind to GT-IIC sequences with higher affinity and repress h*CS* promoter more efficiently than TEF-1α. These results suggest that each TEF-1 isoform may play unique regulatory roles in various tissues.

Transcription enhancer factor-1, TEF-1,[1] is the prototype of four human TEA/ATTS DNA binding domain genes that includes TEF-3, TEF-4, and TEF-5. These homologues are derived from separate genes, and the individual products are expressed differentially in a variety of tissues (*1*−*5*). Initially TEF-1 was identified as a 53 kDa HeLa protein required for SV40 early gene transcription (*1*), which is mediated by TEF-1 binding to the GT-IIC and SphI/SphII enhansons associated with the SV40 early gene enhancer (*2*, *6*, *7*). Subsequent studies proved TEF-1 is a ubiquitous factor implicated in directing the expression of a wide variety of genes, including human papillomavirus-16 *E6* and *E7* oncogene transcription (*8*), murine cardiac and skeletal muscle-specific gene expression (*9*−*13*), and early gene expression in mouse development (*14*). Initially it was thought that TEF-1 was the major factor mediating control of human chorionic somatomammotropin (h*CS*) gene expression in the placenta through its actions on a placental-specific enhancer (*15*−*18*). Subsequent studies have implicated another member of the TEF family, TEF-5, in this placental-specific regulation (*4*, *5*), although the exact roles of these factors remain to be elucidated. The *Drosophila* homologue of TEF-1, scalloped, is involved in sensory organ differentiation (*19*) and wing development (*20*, *21*). TEF-3 has been implicated in muscle-specific gene expression (*3*), and TEF-4 appears to be involved in central nervous system development (*3*).

TEF-1 is composed of several evolutionarily conserved domains, including the TEA/ATTS DNA binding domain, proline-rich, serine-threonine-tyrosine-rich, and zinc finger-like domains (*2*, *7*, *22*, *23*). The TEA/ATTS domain was originally identified as a region of homology between the yeast TEC1 (*22*), TEF-1, and the *Aspergillus nidulans* factor Aba (*23*). In these factors, the 68 amino acid TEA/ATTS domain is located toward the amino terminus of the protein and contains 3 putative γ-helices, 2 of which have been demonstrated to be important for DNA binding by mutational analysis (*7*). A GGAATG consensus binding sequence has been determined both by alignment of natural binding sites (*1*, *2*) and by binding site selection assays using oligonucleotides containing random sequences (Jiang and Eberhardt, unpublished data).

The mechanism of TEF-1 action appears to be complex. Overexpression of TEF-1 in a variety of cells resulted in squelching of transcriptional activity (*2*, *7*, *8*, *24*), suggesting that other limiting transcriptional co-activators are required for TEF-1 transactivation functions. We and others subsequently found that TEF-1 can interact with TBP (*6*, *24*, *25*), an essential component of the basal transcription machinery, and prevent TBP binding to the TATA box (*24*). These

[†] Supported by National Institutes of Health Grant DK51492.

* Address correspondence to this author at the Endocrine Research Unit, 5-194 Joseph, Mayo Clinic, 200 First St. SW, Rochester, MN 55905. Phone: 507-255-6554, FAX: 507-255-4828, E-Mail: eberhardt@mayo.edu.

[‡] Endocrine Research Unit.

[§] Department of Biochemistry/Molecular Biology.

[1] Abbreviations: TEF, transcription enhancer factor; CS, chorionic somatomammotropin; GH, growth hormone; TBP, TATA binding protein; LUC, luciferase; DTT, dithiothreitol; poly(dI-dC), poly(deoxyinosine-deoxycytosine); PMSF, phenylmethylsulfonyl fluoride.

10.1021/bi991048w CCC: $19.00
Published on Web 02/25/2000

findings provide one mechanism by which TEF-1 may negatively regulate gene expression. More recently, two other proteins that are capable of interacting with TEF-1 appear to be important in regulating gene transactivation. Gupta et al. (*26*) have demonstrated that TEF-1−Max interactions are important for γ-myosin heavy chain gene regulation in heart muscle. Also, the *Drosophila* TEF-1 homologue scalloped has been found to interact with the *vestigial* gene product, where it has been shown to regulate wing-specific gene expression (*20*, *21*).

In the current studies, we have cloned and characterized two novel TEF-1 isoforms from a pancreas cDNA library that appear to arise as a result of alternative splicing. These novel isoforms are expressed in unique tissue-specific expression patterns and exhibit differential functional activities in DNA binding and transactivation/transrepression assays. The data suggest that these unique TEF-1 isoforms may play unique regulatory roles in selected tissues.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized in the Molecular Biology Core Facility, Mayo Clinic. Dithiothreitol (DTT), poly(deoxyinosine-deoxycytosine) [poly(dI-dC)], and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Bio-Gel P-100, 30% Acrylamide/Bis (29:1) solution, and 4−15% sodium dodecyl sulfate (SDS) gradient gels were purchased from BIO-RAD (Richmond, CA). [γ-^{32}P]ATP (5000 Ci/mmol) and [^{35}S]-methionine (1200 Ci/mmol) were purchased from Amersham Life Science, Inc. (Arlington Heights, IL).

Gel Shift Assay. Double-stranded wild-type and mutant SV40 GT-IIC oligonucleotides (Table 1) were used as probes to evaluate DNA binding activities of TEF 1 isoforms. Probe preparation, binding reaction conditions, and gel electrophoretic analyses were carried out as previously described (*27*). For thermostability measurements, the in vitro translated TEF-1 proteins were exposed to the designated temperature for 2 min in a PCR cycler. Gels were dried in vacuo and exposed to Kodak X-ray film at 80 °C with intensifying screens for 8−36 h. In vitro translation and ^{35}S-labeling of the TEF-1 isoforms were accomplished using the TNT in vitro transcription translation system (Promega, Madison, WI) according to the manufacturer's specifications. TEF-1α, TEF-1β, and TEF-1γ cDNA subcloned in *pXJ40* vector were used as template. All the reactions were directed by T7 RNA polymerase. Radioactive TEF-1 proteins were produced in reactions containing ^{35}S-labeled methionine and an amino acid mixture lacking methionine (Life Technologies, Inc., Gaithersburg, MD). To evaluate whether comparable amounts of the three isoforms were generated from in vitro transcription translation reactions, 3 μL of the respective products and 10 μL of prestained protein standards (Klaidoscope, BIO-RAD, Richmond, CA) were resolved in 4−15% SDS gradient gels (BIO-RAD). Gels were dried in vacuo and exposed to Kodak X-ray film at room temperature with intensifying screens for 6−18 h.

Cell Transfection and Luciferase Assays. BeWo human choriocarcinoma cells (ATCC, Rockville, MD) were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD). All media were additionally supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 100% humidity. Cell harvesting, transfection, and luciferase assays were performed as described previously (*17*) except that 5 μg of reporter plasmid and various amounts of TEF-1 isoform expression plasmid or control *pBluescript* (Stratagene, San Diego, CA) plasmid DNA were used. Optimal electroporation conditions for both BeWo and AsPC cells were 250 V at 960 μF. All plasmid DNAs were isolated by large-scale alkaline lysis and purified by double CsCl gradient ultracentrifugation.

Plasmids. All the luciferase reporter constructs were based on *pA3LUC* (*28*). Construction of *CSp.LUC*, containing 498 bp promoter, and *$(EnA)_2$_CSp.LUC*, containing both *CS* promoter and 2 copies of the minimal 240 bp *CS* enhancer, was described previously (*17*). *$(GT\ IIC)_{24}$_CSp.LUC* contains 12 tandem GT-IIC repeats subcloned in *CSp.LUC* at the same position as the CS enhancer (*27*). *pXJ40-TEF-1A* eukaryotic expression plasmid (*2*) containing the prototypic TEF-1, which we designate as TEF-1α, was generously provided by Dr. Pierre Chambon (University of Strasbourg, Strasbourg, France). To construct TEF-1β and TEF-1γ eukaryotic expression plasmids, the corresponding β- and γ-specific regions were replaced in *pXJ40-TEF-1A* plasmid by first digesting the intact plasmid with *Eco*RI−*Bam*HI to remove a ca. 500 bp fragment corresponding to the 5′-end of the TEF-1α cDNA. After gel electrophoresis and purification with GENECLEAN, this fragment was digested with *Bfa*I to generate a 270 bp *Eco*RI/*Bfa*I fragment, corresponding to the extreme 5′-end of the TEF-1α cDNA. This fragment was subsequently purified by gel electrophoresis and GENECLEAN and used as a PCR primer along with TEF-1βBam primer (Table 1) with the TEF-1β and TEF-1γ cDNAs as templates. The resulting PCR products extended the β and γ cDNAs to replace the upstream conserved regions. These products were digested with *Eco*RI and *Bam*HI and subcloned into *pXJ40-TEF-1A* digested with the same enzymes. The clones were sequenced to verify the structures. Both isoforms exhibited the correct β- and γ-isoform-specific DNA sequences but contained random PCR-generated mutations in downstream conserved regions. These mutations were replaced by digesting these plasmids with *Pst*I and replacing the excised 290 bp fragment with the 290 bp *Pst*I fragment from the corresponding region of *pXJ40-TEF-1A.*

Cloning and PCR Detection of TEF-1β and TEF-1γ from Multiple Tissues. TEA domain-related sequences were PCR-amplified by the degenerate primers TIC.11 and TIC.12 (Table 1, Figure 1A). QUICK-Screen human library panel (CLONTECH, Palo Alto, CA) and Marathon-Ready human cDNA panel (CLONTECH) were used as sources of cDNA templates from multiple tissues. PCR was carried out using an Expand long-range PCR system (Promega, Madison, WI) with *Taq* polymerase. After initial denaturation at 94 °C for 10 min, 30 cycles of amplification were performed as follows: 94 °C for 1 min denaturation followed by 50 °C for 1.5 min annealing and 72 °C for 2 min extension. After completion of the amplification, there was a final extension performed at 72 °C for 10 min. PCR products were purified by phenol/chloroform extraction, concentrated by ethanol precipitation, treated with T4 DNA polymerase, and sub-

Table 1

name	DNA sequence[a]
GT-IIC$_{SV}$(+)	GCAGCTGTGGAATGTGTGTCTC
GT-IIC$_{SV}$(−)	GAGACACACATTCCACAGCTGC
GT-IIC$_{SV}$MUT(+)	GCAGCCTAGCGTGACTGGTCTC
GT-IIC$_{SV}$MUT(−)	GAGACCAGTCACGCTAGGCTGC
TEF-1βBam	GAGGATCCTGGCTGCCCTGTTTGAATG
TIC.11	GGTAGATCTGAYGARGGNAARATGTAYGG
TIC.12	GGTAGATCTRTTCATCATRTAYTTYTC
TEF-1.AS1	GGCGAATTCGAGAAATTCAAGCCGCCA
TEF-1.AS2	GGCGAATTCGCCGCCATTAAGGTAAC
TEF-1.101	GGCGAATTCGCTCAAACCCTGGAATGG

[a] R = G and A; Y = T and C; N = G and A and T and C.

cloned into *PCR-Blunt* vector (Invitrogen, Carlsbad, CA). Positive clones were sequenced to identify novel TEA domain factors. To examine the tissue distribution of the individual TEF isoforms, specific primers capable of detecting both TEF-1β and TEF-1γ (TEF-1.AS1) or TEF-1γ only (TEF-1.AS2), respectively, were used in combination with a common downstream primer (TEF-1.101) (Table 1, Figure 1A). PCR products were examined by electrophoresis in 1.5% agarose gels and subsequent ethidium bromide staining.

Data Analysis. All data groups were analyzed by multivariate analysis of variance (ANOVA) to determine if the TEF-1 isoforms' effects on reporter activities were significant at the $p < 0.05$ level. The results of the ANOVA are provided in the figure legends. For all experimental groups which satisfied the initial ANOVA criterion, individual comparisons were performed with the use of post hoc Bonferroni *t* tests. The controls for the comparison are designated in the figure legends.

RESULTS

Molecular Cloning of Novel TEF-1 Isoforms. Several lines of evidence have suggested that multiple TEF isoforms are present in placenta and other tissues. First, in efforts to clone other members of the TEF family, several clones containing insertion and deletions were isolated from a human placental cDNA library (Jiang and Eberhardt, unpublished data). Second, multiple bands were observed in PCR products of TEA/ATTS domain-containing genes (Jiang and Eberhardt, unpublished data). Finally, two differential splicing forms of the chicken TEF-5 homologue (DTEF-1) have been described (*29*). To understand the expression and function of these isoforms, we set out to clone them systematically using the degenerate PCR primers TIC.11 and TIC.12 (Table 1) which span highly conserved regions in all the known TEFs (see Figure 1A). Either QUICK-Screen human library panel (CLONTECH) or Marathon-Ready human cDNA panels (CLONTECH), representing cDNA from placenta, pancreas, brain, lung, liver, kidney, ovary, prostate, and lymphoid tissues, were used as sources of cDNA templates. PCR products were cloned and sequenced. In addition to many clones representing the original TEF-1 cloned by Xiao et al. (*2*), which was redesignated TEF-1α in the current studies, two novel clones, designated TEF-1β and TEF-1γ, were identified from pancreas and placenta. These two clones were identical to TEF-1α except for the region immediately downstream of the TEA/ATTS domain (Figure 1A), designated the hinge region. TEF-1β contains 19 nucleotide substitutions in a 60 nucleotide span, leading to 8 amino acid alterations (Figure 1B,C). TEF-1γ contains the same substitutions as TEF-1β and an additional 12 nucleotide insert following the substitutions (Figure 1B,C).

In Figure 1C we have compared the amino acid sequences of the human isoforms with all the other known TEF isoforms in human, mouse, and chicken. From this analysis, it appears that the hTEF-1γ and the mouse TEF-1 variant cloned by Shimizu et al. (*7*) are related, since they both contain VTSM inserts in the hinge region; however, these TEF-1 isoforms define two classes defined by the amino acid sequences between the terminus of the TEA/ATTS domain and the VTSM insert. Like hTEF-1α, all of the mouse isoforms contain the sequence RDFHS in this region. In contrast, the human β and γ isoforms have the sequence REIQA, which is identical to the cTEF-1 and TEF-3 isoforms. Interestingly, the cTEF-1c isoform also contains a VTSM insert albeit at a position about 100 amino acids downstream of the TEA/ATTS domain (Figure 1C). The conservation of this motif among the TEF-1 isoforms of all species examined to date suggests that this may have special functional significance. While the origin of these isoforms is not known, they most likely arise from a single gene by mutually exclusive splicing (Discussion). Potential splicing sites that conform to exon consensus sequences bounding introns are indicated in Figure 1B and bracket the region containing the altered nucleotides in each of the isoforms. Like the case with TEF-1γ, hTEF-5 also contains an insert of four amino acids (AMNL) in the same hinge region, suggesting that other TEF-5 isoforms may also exist (Figure 1C).

TEF-1α has been well established as an ubiquitously expressed factor found in lungs, liver, brain, muscle, and placenta (*3*). We examined the tissue-specific expression pattern of the newly identified TEF-1β and TEF-1γ mRNAs, using PCR to generate reliable isoform-specific signals for TEF-1β and TEF-1γ. Unlike TEF-1α, TEF-1γ mRNA showed a very limited tissue expression pattern. From seven major tissues examined, TEF-1γ was only detected in heart and skeletal muscle, pancreas, and kidney, whereas TEF-1β appeared to be detectable in all tissues except liver (Figure 2A,B). The different tissue-specific expression patterns suggest these isoforms may play distinct transcriptional regulatory roles in various tissues.

TEF-1 Isoforms Differ in Their DNA Binding Activities. Since the amino acid substitutions and insertions occur immediately downstream of the TEA/ATTS domain, these isoforms may have altered DNA binding activities. Consequently, their abilities to bind to the consensus GT-IIC sequences were evaluated by gel-shift assays using in vitro translated proteins. To ensure that equal amounts of the different proteins were used for these comparisons, each isoform was transcribed from the same vector, and the proteins were labeled with [^{35}S]methionine during in vitro translation to allow the products to be visualized on SDS−PAGE gels (Figure 3A). Each of the three isoforms migrates as single bands with the expected molecular size of 53 kDa and with equal intensity of [^{35}S]methionine labeling, indicating that the proteins are intact and produced at comparable concentrations, allowing a meaningful comparison of their respective DNA binding activities.

In gel shift assays, all the in vitro translation products form distinct complexes with GT-IIC wild-type but not mutant

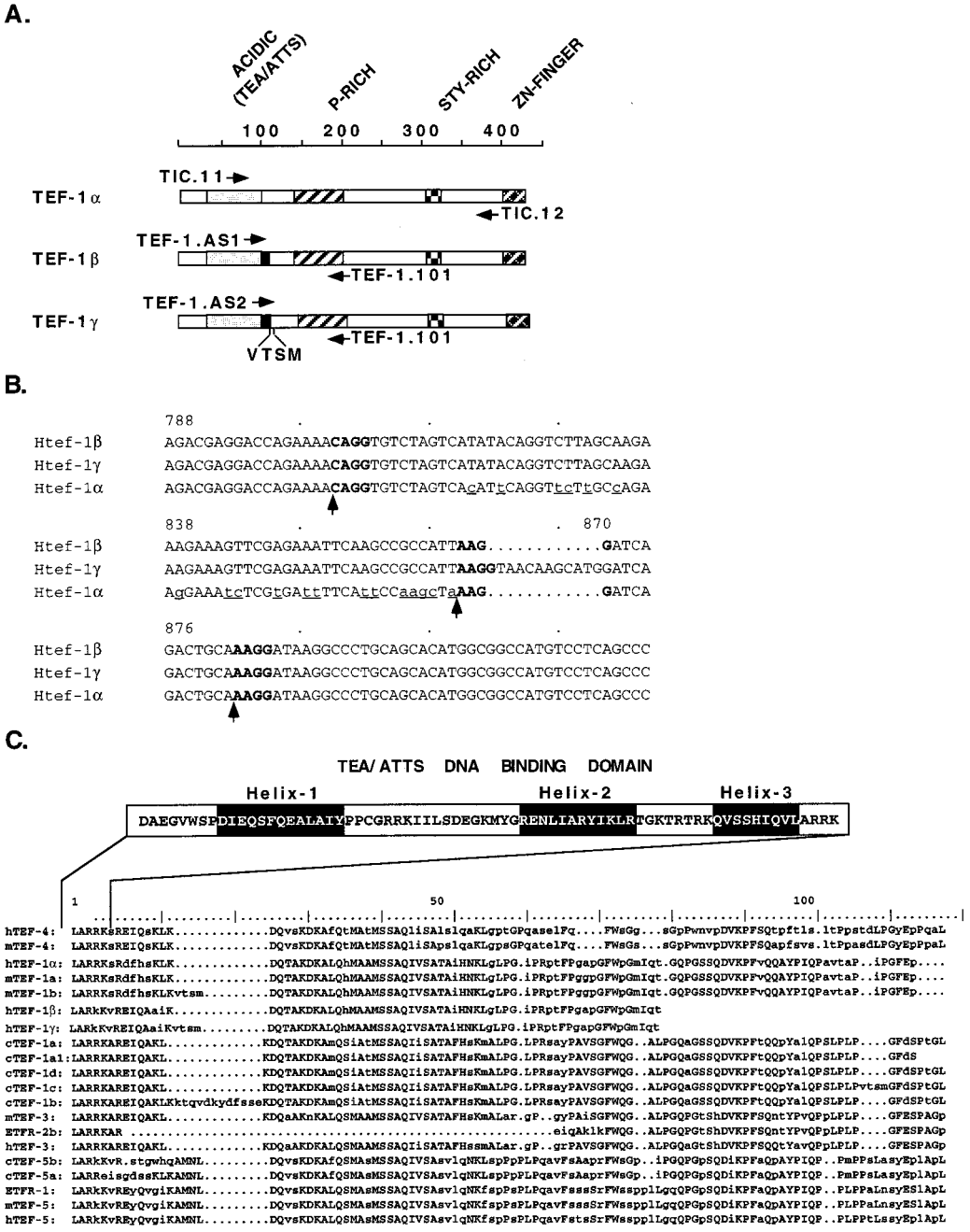

FIGURE 1: Cloning and structure of TEF-1β and TEF-1γ isoforms. (A) Cloning strategy. The degenerate oligonucleotides TIC.11 and TIC.12 (Table 1) corresponding to highly conserved regions within the DNA binding domain (TEA/ATTS, stippled box) and another region upstream from the Zn fingerlike domain (cross-hatched box) were used for PCR amplification. The P-rich (striped box) and STY-rich (checkered box) domains in the TEF-1 homologues are also indicated. TEF-1.AS1 and TEF-1.AS2 represent unique oligonucleotides used to detect specifically the β and γ isoforms; TEF-1.AS1 detects TEF-1β and TEF-1γ simultaneously, and TEF-1.AS2 detects only the TEF-1γ isoform. TEF-1.AS1 and TEF-1.AS2 were used in reverse transcription/PCR assays to determine the isoform's cell-specific distribution in conjunction with the common oligonucleotide primer TEF-1.101. The TIC.11 and TIC12 amplimers yield amplicons of 870 and 882 bp, respectively, for the β and γ isoforms. The amplicons from TEF-1.AS1/TEF-1.AS2-TEF-1.101 amplimers are 284 and 286 bp, respectively, for the β and γ isoforms. (B) Nucleotide sequence comparison of TEF-1α with the TEF-1β and TEF-1γ isoforms. Numbering conforms to TEF-1α (GenBank Accession No. M63896). Nucleotides in boldface type represent putative splice site boundaries that conform to most probable exon consensus sequences that bound introns (*47*). Nucleotides that differ are indicated in lower case letters and are underlined. Potential splicing sites conforming to exon consensus sequences (*47*) are shown in boldface, and the putative exon−exon boundaries are marked by arrows. (C) Amino acid sequence comparison of the human TEF-1β and TEF-1γ isoforms with human (h), mouse (m), and chicken (c) TEF-1, TEF-3, TEF-4, and TEF-5 homologues. The numbering is arbitrary. The isoforms, with Genbank Accession Numbers in parentheses, are: hTEF-4 (X94440), mTEF-4 (X94442), hTEF-1α (M63896), mTEF-1a (L06865), mTEF-1b (L13853), cTEF-1a (U04834), cTEF-1a1 (U06848), cTEF-1d (U06851), cTEF-1c (U06850), cTEF-1b (U06849), mTEF-3 (X94441), ETFR-2b (D87965), hTEF-3 (X94438), cTEF-5b (U46128), cTEF-5a (U46127), ETFR-1 (D87963), mTEF-5 (Y16611), and hTEF-5 (AF142482). Amino acids that differ are indicated in lower case letters. The protein sequences were obtained by translation of the nucleotide sequences, and multiple sequence comparison was performed with PILEUP and PRETTY (Genetics Computer Group, Madison, WI).

probe, which contains substitutions in the GGAATG core DNA binding element, indicating that each of the proteins forms specific complexes with DNA (Figure 3B). However, their relative affinities for the wild-type GT-IIC DNA probe

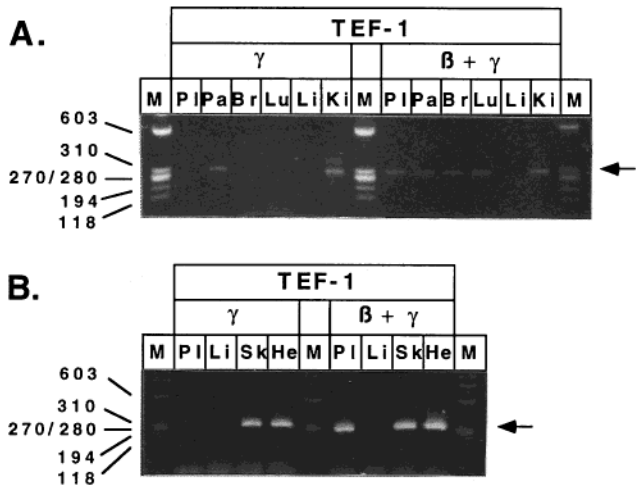

FIGURE 2: Tissue-specific distribution of the TEF-1β and TEF-1γ isoforms. (A) Reverse transcription and PCR amplification of total RNA from placenta (Pl), pancreas (Pa), brain (Br), lung (Lu), liver (Li), and kidney (Ki) were performed as described under Materials and Methods using the TEF-1β- and TEF-1γ-specific oligonucleotides TEF-1.AS1 and TEF-1.AS2 with the common primer TEF-1.101. (Table 1, Figure 1A). (B) Reverse transcription and PCR amplification of total RNA from placenta (Pl), liver (Li), skeletal muscle (Sk), and heart muscle (He) were performed as described under Materials and Methods using the TEF-1β- and TEF-1γ-specific oligonucleotides TEF-1.AS1 and TEF-1.AS2 with the common primer TEF-1.101 (Table 1, Figure 1A). ΦX174 replicative form DNA digested with *Hae*III restriction endonuclease served as the size marker (M).

differ significantly, in the order: TEF-1γ > TEF-1β > TEF-1α. Interestingly, while TEF-1β and TEF-1γ complexes migrated to a very similar position, both migrated somewhat faster than TEF-1α (Figure 3B,D). Identical results were observed in three different experiments using independently generated in vitro translation products (data not shown). Such mobility differences may be due to differences in these isoforms' conformational states.

To provide a quantitative estimate of the differences in the TEF isoforms' DNA binding affinities, we performed titration experiments in DNA binding reactions using increasing amounts of in vitro translation products with a constant amount of the GT-IIC probe. After gel resolution, the specific DNA−protein complexes were scanned and subjected to densitometry analysis. These data confirm the relative binding order of the three isoforms observed above (Figure 3C) and indicate that TEF-1β and TEF-1γ have ca. 2.1- and 4.7-fold higher affinity, respectively, than TEF-1α to the GT-IIC enhanson.

Earlier studies indicated that every GT-IIC binding factor has its characteristic thermostability profile (*27*). Thermostabilities of newly identified TEF-1 isoforms were consequently characterized (Figure 3D). Heat inactivation of TEF-1α appeared to be a gradual process from 55 to 60 °C; detectable DNA binding activity remains after 65 °C treatment; TEF-1β and TEF-1γ DNA binding activities were similarly inactivated after heat treatment at 60 °C. Unlike TEF-1α, both the β and γ isoforms demonstrated detectable loss of binding activity at 55 °C, and no binding activity could be detected after treatment at 65 °C. We conclude that the limited amino acid substitution and insertion in TEF-1 isoforms caused substantial divergence in their DNA binding affinities, DNA−protein complex migration, and thermostabilities. These data suggest that the region immediately downstream of the TEA/ATTS domain may contribute to DNA binding activity, possibly by altering protein conformation and modulating the adjacent DNA binding domain.

TEF-1 Isoforms Exhibit Differing Transrepressor Activities. In previous studies, we have shown that TEF-1α overexpression inhibited the h*CS* and a variety of other promoters in BeWo cells (*24*). Such inhibition did not depend on the presence of TEF-1α binding sites. Based on in vitro protein retention assays, we demonstrated that TEF-1α can interact with TBP, a component of the basal transcriptional apparatus, and inhibit TBP binding to the TATA box (*24*). While the physiologic relevance of this inhibition has yet to be established, this characteristic of TEF-1 overexpression on functional activity has been reproduced in many laboratories and was used to evaluate the transcriptional activities of the novel TEF-1 isoforms. Thus, we subcloned TEF-1β and TEF-1γ into the same expression vector as TEF-1α and cotransfected the luciferase reporter constructs with the three TEF-1 isoform expression vectors. The *CSp.LUC* expression vector, which contains the 498 bp h*CS* promoter, was used to compare the promoter repression potential of the isoforms. Also, in previous studies, we have demonstrated that multiple TEF-1 binding sites present on the h*CS-2* gene enhancer are essential for its enhancer activity. Consequently, the *(EnA)$_2$_CSp.LUC* construct, containing the h*CS* promoter and two copies of the h*CS-2* enhancer, was used to explore the possible role that the TEF-1 isoforms may play on *CS* gene expression. Since the native *CS* enhancer is composed of multiple enhansons in addition to GT-IIC, we employed *(GT-IIC)$_{24}$_CSp.LUC*, a plasmid carrying 24 copies of the GT-IIC consensus sequence, to confirm the transcriptional activities of TEF-1 isoforms by excluding the interference of non-GT-IIC enhansons in the native enhancer.

The transfection data indicated that TEF-1β and TEF-1γ are transcriptional repressors (Figure 4A−C). Two interesting findings, however, resulted from detailed analysis of the data. First, the three TEF-1 isoforms have different transrepressor activities. At subsaturating TEF-1 concentrations (40 and 200 ng), TEF-1γ is significantly more efficient than TEF-1α. Second, all the isoforms showed stronger repression on *(GT-IIC)$_{24}$_CSp.LUC* than *CSp.LUC*, suggesting TEF-1 binding to DNA may contribute to its transrepression (Figure 4C).

DISCUSSION

In the current studies we have cloned and characterized two novel isoforms of the prototypic TEF-1 (TEF-1α) that were designated TEF-1β and TEF-1γ. Both isoforms contain sequence alterations in a region just downstream from the highly conserved TEA/ATTS DNA binding domain. From a comparison of the human TEF-1β and TEF-1γ isoforms with the other TEF isoforms from human, mouse, and chicken, it is apparent that the TEF-1 isoforms fall into two classes defined by the amino acid sequences just downstream from helix-3 in the C-terminal region of the TEA/ATTS domain (Figure 1C). Helix-3 and helix-1 have been shown to be important for DNA binding (*7*). Thus, like human TEF-1α, the two mouse TEF-1 isoforms contain the sequence RDFHS downstream from helix-3. In contrast, the human β and γ isoforms possess the sequence REIQA, which is identical to the cTEF-1 and TEF-3 isoforms. In addition, the

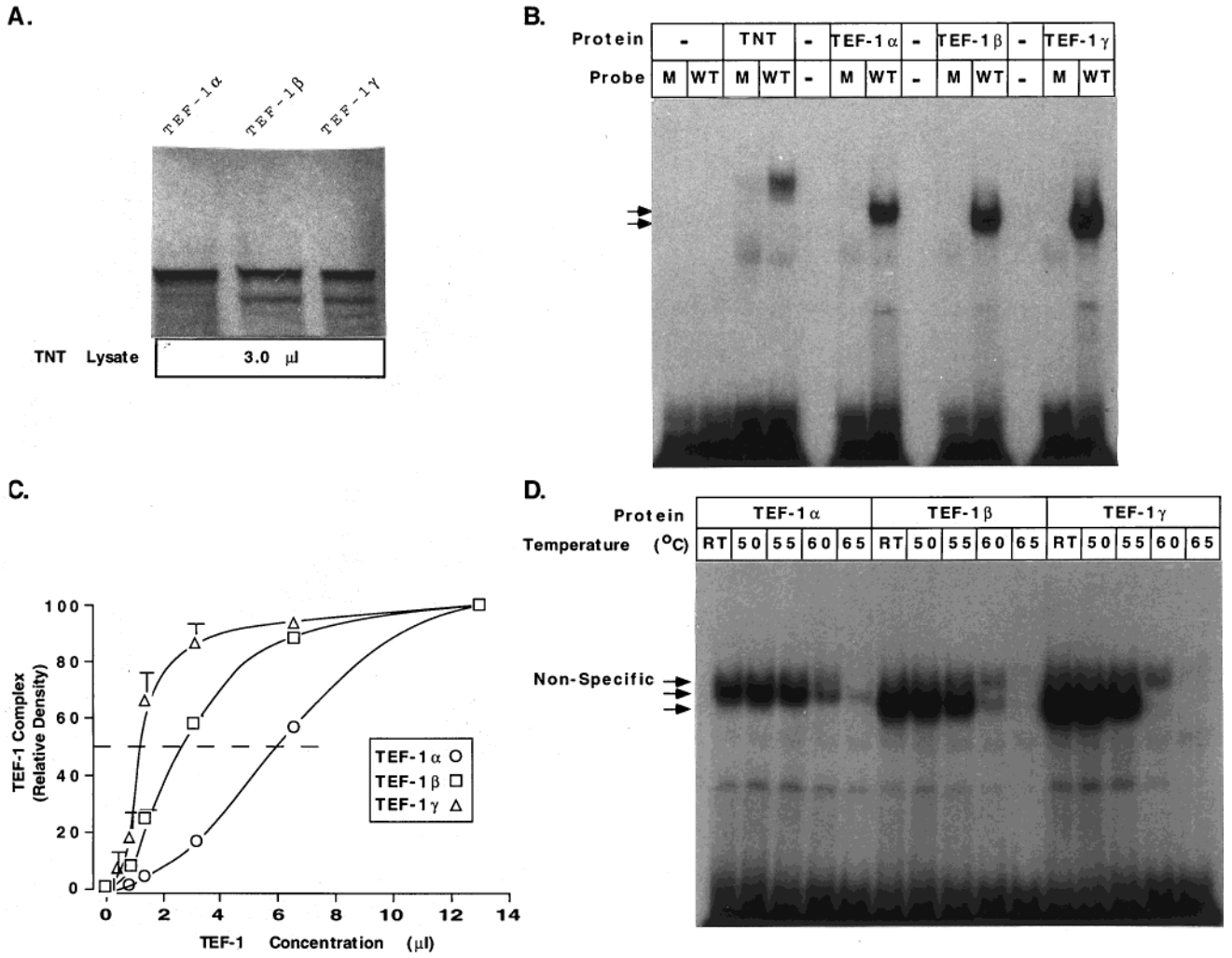

FIGURE 3: Comparison of the DNA binding properties of the TEF-1β and TEF-1γ isoforms. (A) Autoradiogram of in vitro translated [^{35}S]methionine-labeled TEF-1α, TEF-1β, and TEF-γ separated by SDS gel electrophoresis in the presence of 2-mercaptoethanol. (B) Gel shift analysis of the binding of TEF-1α, TEF-1β, and TEF-γ to wild-type (WT) and mutant (M) GT-IIC oligonucleotides (Table 1). The TNT lysate without DNA template was used as an additional control. Arrows indicate the relative migration of the various complexes. (C) Densitometric analysis of the binding of increasing concentrations of TEF-1α, TEF-1β, and TEF-γ to the wild-type GT-IIC oligonucleotide. Assays were performed as described under Materials and Methods. The data represent the averaged results from two independent experiments. Error bars represent the average variation of the mean between the two experimental determinations. The relative affinities were estimated from the differences in protein concentration required to achieve 50% of the maximum binding (dashed line). (D) Thermostability of the TEF-1α, TEF-1β, and TEF-γ isoforms. The proteins were heated for 2 min at the indicated temperatures for 2 min prior to incubation in standard gel shift binding reactions with the wild-type GT-IIC oligonucleotide (Materials and Methods). Specific protein−DNA interactions are indicated by arrows.

chicken TEF-1 isoforms are more similar to the TEF-3 isoforms in sequences further downstream from the TEA/ATTS domain (Figure 1C, positions 40−80). Interestingly, the TEF-1β and TEF-1γ isoforms, which bear some resemblance to TEF-3 through the REIQA motif, are also expressed abundantly in heart and skeletal muscle, where TEF-3 is known to be important, suggesting that these isoforms may have special significance in these tissues. Since the human TEF-1 isoforms contain both classes represented by the RDFHS and REIQA motifs, it seems likely that a number of other variants that correspond to these two classes may exist.

Since TEF-1β and TEF-1γ share 100% identity with TEF-1α in both the 5′- and 3′-regions from the site of the nucleotide substitutions and a single human TEF-1 gene has been localized to chromosome 11p15.2 by fluorescence in situ hybridization and PCR screening of hybrid panels (*30*), the TEF-1 isoforms are most likely derived from alternative splicing of TEF-1 pre-mRNA. Alternative splicing is a common scheme to generate transcriptional factor isoforms with diverse functions in a tissue-specific manner (*31*, *32*). In the current study, the distinct tissue-specific expression pattern and differential transcriptional activities of TEF-1α, TEF-1β, and TEF-1γ suggests that these isoforms may control the expression of different genes and/or the same set of genes in different ways.

Alternative splicing can occur in several different ways. Use of alternate splice acceptor sites within exons can lead to the deletion of specific protein-encoding regions. A classical example is represented by the 20 kDa variant of human growth hormone (*33*). In contrast, alternative acceptor sites within an intron lead to the insertion of novel coding regions, as in the case of the alternative splice acceptor site in intron 1 of the human GHF-1/Pit-1 mRNA. This event generates GHF-2/PIT-2 mRNA with a 78 bp in-frame insertion upstream from exon 2 (*34*, *35*). Another type of

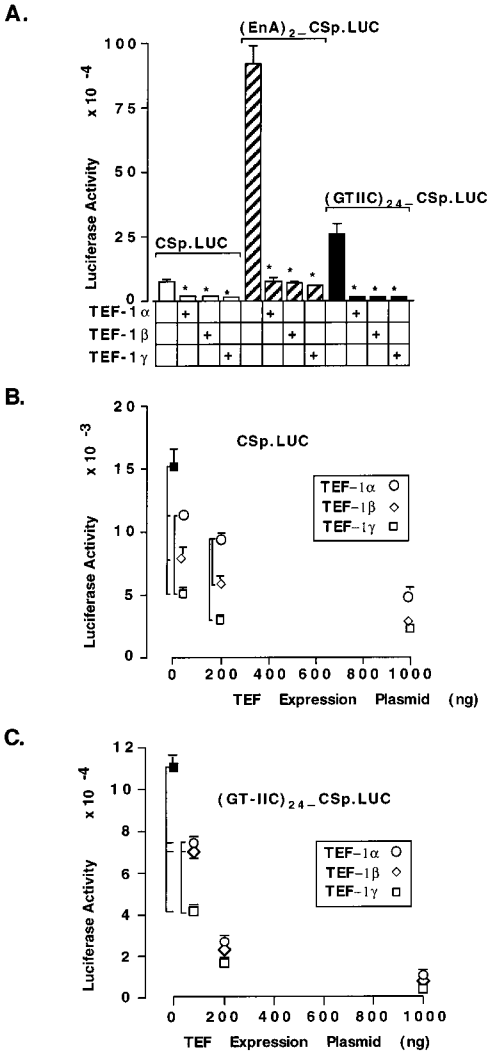

FIGURE 4: Comparison of the functional activities of the TEF-1β and TEF-1γ isoforms. (A) Inhibition of h*CSp.LUC*, *(EnA)$_2$_CSp.LUC* (wild-type enhancer), and *(GT-IIC)$_{24}$_CSp.LUC* (synthetic enhancer) activity in BeWo cells. Cells were transfected as described under Materials and Methods with 1 μg of the TEF-1α, TEF-1β, and TEF-1γ plasmid DNA. Asterisks denote significant post hoc Bonferroni *t* test values ($p < 0.05$) to compare individual differences among the multiple variables. Multivariate ANOVA results were significant at the $p < 0.0001$ level. (B) Concentration dependence of the inhibition of h*CS* promoter activity in BeWo cells. Cells were handled identically as in (A) except that 40, 200, or 1000 ng of TEF-1α (open circles), TEF-1β (open diamonds), and TEF-1γ (open squares) plasmid DNA was used in these transfections. The h*CS* promoter activity in the absence of TEF-1 is indicated by the solid square. The data represent the results of 4 independent transfections. Error bars represent the standard errors. The data were analyzed by multivariate ANOVA ($p < 0.0001$) and post hoc Bonferroni *t* tests ($p < 0.05$) which are indicated by the individual data connected with the brackets. (C) Concentration dependence of the inhibition of the synthetic GT-IIC enhancer-mediated activity in BeWo cells. Cells were handled identically as in (A) and (B) using 40, 200, or 1000 ng of TEF-1α (open circles), TEF-1β (open diamonds), and TEF-1γ (open squares). Plasmid DNA was used in these transfections. The GT-IIC enhancer driven h*CS* promoter activity in the absence of TEF-1 is indicated by the solid square. The data represent the results of 4 independent transfections. Error bars represent the standard errors, and the data connected by brackets indicate the statistically significant differences at the $p < 0.05$ level as determined by multivariate ANOVA ($p < 0.0001$) using a post hoc Bonferroni *t* test to compare individual differences among the multiple variables.

alternative splicing takes the form of whole exon insertion or skipping. The neurofibromatosis 1 (*NF1*) gene contains two alternatively spliced exons (23a and 48a) and provides an example in which both exon insertion and skipping are observed. Type 1 *NF1* mRNA contains neither 23a or 48a exon sequences, whereas type 2 *NF1* mRNA contains only the 23a exon insertions (*36*). Numerous examples of trans-splicing between two different pre-mRNAs is known to occur frequently in plants and protists; however, it is not known whether this form of splicing plays a significant role, if at all, in mammalian mRNA splicing (*37, 38*). Finally, another form of splicing which appears to be widespread is mutually exclusive splicing. In this form of splicing, two or more related exons are incorporated in the same position in a mutually exclusive manner. The related exons appear to have evolved by exon-specific gene duplication events and subsequent divergence (*39*−*44*). As discussed below, this form of splicing represents the most likely form of alternative splicing to generate the γ, β, and γ TEF-1 transcripts.

Several examples of mutually exclusive splicing are known to generate functionally important and distinct isoforms, including β-tropomyosin (*39*−*41*), cardiac calcium channel γ1 subunit (*42*), the voltage-gated SCN8A sodium channel (*43*), and synaptosomal associated protein SNAP-25 (*44*). While the mechanisms accounting for exclusive splicing are not completely understood, studies of β-tropomyosin alternative splicing indicate that this is a tightly regulated process. Using mutation analysis, Libri et al. (*39*) showed that exon and intron sequences act in cis to regulate incorporation of exon 6A in a cell-specific manner in myoblast or smooth muscle tissues and exon 6B into skeletal muscle or cultured myotubes. These studies identified a polypyrimidine tract that was important for mediating the cell-specific splicing, and subsequent studies have established that these sequences act as an intronic enhancer that is essential for exon 6A 5′-splice site recognition (*45*). More recently, the splicing recognition (SR) factors ASF/SF2 and SC35 have been shown to stimulate and inhibit, respectively, exon 6A splicing in smooth muscle cells (*40*), and a separate G-rich enhancer has been identified with exon 6B exclusive splicing (*46*). Accordingly, it has been proposed that the relative levels of ASF/SF2 and SC35 may control the tissue-specific splicing associated with exon 6A (*45*).

While structural information about the human TEF-1 gene is lacking, several factors provide strong support for the concept that mutually exclusive splicing accounts for the γ, β, and γ TEF isoforms. First, only a single TEF-1 gene has been identified. Second, the fact that the spliced isoforms are highly related and contain variant amino acids randomly distributed over an otherwise conserved region (Figure 1C) is precisely the pattern predicted by exclusive splicing. Also, since the various isoforms exhibited different distribution patterns in the examined tissues (Figure 2), mechanisms similar to those described for β-tropomyosin discussed above may be involved in regulating this cell-specific expression.

TEF-1 DNA binding activity has been characterized by mutation and deletion studies (*7*). Interruption of the first putative γ-helical structure within the TEA/ATTS domain by introduction of proline in the conserved positions completely abolished binding to GT-IIC and SphI/II enhansons. Mutation of the second putative γ-helical structure had no effect on DNA binding, whereas mutation of the third

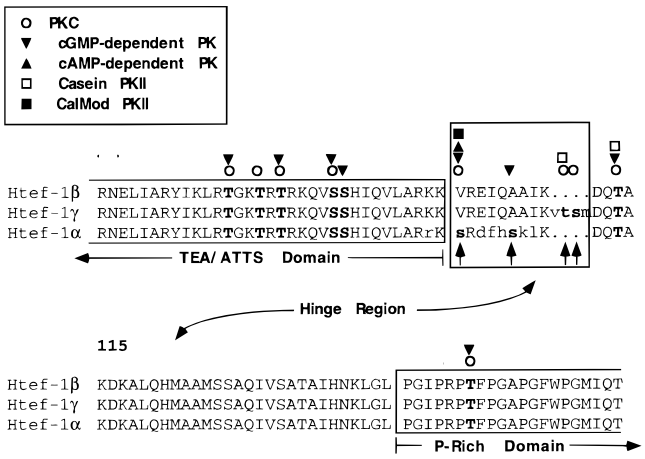

FIGURE 5: Potential phosphorylation sites within the region affected by the amino acid substitutions/insertions among TEF-1 isoforms. Phosphorylation sites are indicated in boldface letters. The different phosphorylation sites are indicated by different symbols: PKC, open circles; cAMP, closed triangles; cGMP, closed inverted triangles; calmodulin-dependent protein kinase II, closed squares; and casein kinase II, open squares. Consensus sequences for the various sites were taken from Pearson and Kemp (*48*).

γ-helical structure resulted in partially diminished DNA binding. These studies emphasize the important role of the TEA/ATTS domain in DNA binding. Nevertheless, deletion of the extreme carboxyl-terminal Zn fingerlike region also caused complete loss of DNA binding activity (*7*). However, further deletion of the carboxyl-terminal region to the end of the STY-rich domain recovered the specific DNA binding, suggesting that strong positive and negative modulators of DNA binding activity are present in the carboxyl-terminal region (*7*). In this study we found that the amino acid sequence alterations downstream of the TEA/ATTS domain caused significant changes in each of the isoforms' DNA binding activities (Figure 3). Since these alterations are remote from any of the identified C-terminal DNA binding modulator domains, this "hinge" region between the DNA binding and P-rich transactivation/transrepression domain (*7*) appears to represent a new DNA binding modulator domain. Interestingly, the DNA binding affinities of the TEF-1 isoforms (TEF-1γ > TEF-1β > TEF-1α) are directly proportional to their transrepression potential (Figure 4). While the "hinge" region is distinct from the P-rich transactivation/transrepression domain (*7*), it may regulate the transcription function of individual isoforms by modulating the DNA binding activity.

How do the alterations in TEF-1 isoforms affect their DNA binding and trans-repressor activities? One possibility is that posttranslational modifications that are specific to each of the isoforms could affect the protein's structure/function. We previously observed that TEF-1α is an in vitro substrate for PKC, and phosphopeptide analysis indicated that the hinge region is phosphorylated (Jiang et al., unpublished observations). Among five consensus PKC phosphorylation sites found in the hinge region adjacent to the TEA/ATTS domain, four of these sites are conserved and one is mutated by the serine to valine replacement at position 102 in the β and γ isoforms (Figure 5). Interestingly, in addition to recognition by PKC, the same residue represents a potential phosphorylation site that can be recognized by calmodulin-dependent protein kinase II and by cAMP- and cGMP-dependent protein kinases. Moreover, the insertion of threonine and serine (VTSM) in TEF-1γ produces two additional potential phosphorylation sites that are recognized by PKC and casein kinase II. Also, since the VTSM insertion is preserved in TEF-1 isoforms in both mouse and chicken, this motif may have particular functional importance. Whether or not phosphorylation mediates some of the documented functional changes in β and γ TEF-1 isoforms will require further study.

ACKNOWLEDGMENT

We thank Dr. Bryan McIver for many helpful suggestions and for encouragement. We are especially indebted to Dr. Alexandre Stewart, University of Pittsburgh, for providing corrected protein translations of the chicken TEF-1 isoforms (a−d) to correct errors in the nucleotide sequences of the GenBank databases (Accession Numbers: U04834, U06848, U06851, U06850, and U06849).

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BI991048W