Regulation of the MiTF/TFE bHLH-LZ transcription factors through restricted spatial expression and alternative splicing of functional domains

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ABSTRACT

The MiTF/TFE (MiT) family of basic helix–loop–helix leucine zipper transcription factors is composed of four closely related members, MiTF, TFE3, TFEB and TFEC, which can bind target DNA both as homo- or heterodimers. Using real-time RT–PCR, we have analyzed the relative expression levels of the four members in a broad range of human tissues, and found that their ratio of expression is tissue-dependent. We found that, similar to the MiTF gene, the genes for TFEB and TFEC contain multiple alternative first exons with restricted and differential tissue distributions. Seven alternative 5' exons were identified in the TFEB gene, of which three displayed specific expression in placenta and brain, respectively. A novel TFEC transcript (TFEC-C) encodes an N-terminally truncated TFEC isoform lacking the acidic activation domain (AAD), and is exclusively expressed in kidney and small intestine. Furthermore, we observed that a considerable proportion of the TFEC transcripts splice out protein-coding exons, resulting in transcription factor isoforms lacking one or more functional domains, primarily the basic region and/or the AAD. These isoforms were always co-expressed with the intact transcription factors and may act as negative regulators of MiTF/TFE proteins. Our data reveal that multiple levels of regulation exist for the MiTF/TFE family of transcription factors, which indicates how these transcription factors may participate in various cellular processes in different tissues.

INTRODUCTION

The basic helix–loop–helix leucine zipper (bHLH-LZ) transcription factors MiTF, TFE3, TFEB and TFEC comprise a family (MiTF/TFE or MiT family) of closely related proteins that bind DNA as homo- and heterodimers (1). All possible combinations of DNA-binding MiTF/TFE dimers have been shown to exist in vitro (1–3).

The members of this family are believed to be implicated in pivotal developmental and cellular processes in various cell types. MiTF is involved in the maturation of melanocytes of neural crest origin, retinal pigment epithelium, and bone marrow-derived mast cells and osteoclasts (4); whereas TFEB is essential for placental vascularization (5). TFE3 plays a role in TGF-β-activated signal transduction (6,7) and B-cell activation (8), and cooperates with MiTF and TFEC in osteoclast development (9–11). Furthermore, chromosomal translocations involving the TFE3 and TFEB genes have been implicated in subtypes of renal cell carcinomas in children and young adults. In addition, TFE3 rearrangements were found in alveolar soft part sarcomas (12–19).

The apparent distinctive cellular functions of MiTF/TFE family members in the various cell types require regulatory mechanisms that tightly control MiTF/TFE functioning. Such mechanisms may involve interactions with cell type-specific factors or specific protein modifications, but the relative levels of co-expression of the various transcription factors may be important as well. Indeed, the expression levels of each of the four MiTF/TFE family members appear to differ considerably between cell types (3,20,21), which points towards a model in which the ratio of MiTF/TFE expression dictates specific dimerization patterns and, subsequently, the expression of target genes. Although there are indications that heterodimeric interactions are not essential for proper MiTF/TFE functioning (11), MiTF–TFE3 and MiTF–TFEC heterodimers do exist in vivo, and are believed to be involved in the regulation of cell type-specific genes (10,21,22).

The MiTF gene is expressed in different isoforms that are under the control of distinct promoters (23,24). Currently, at least eight major MiTF isoforms have been identified that are differentially expressed in a variety of tissues, including melanocytes, heart and mast cells (4,21,24–28). These isoforms share the important functional domains of MiTF, including the transactivation domain, basic region, helix–loop–helix and leucine zipper, but differ in their N-termini. These N-termini may contribute to the cell type-specific properties of the various isoforms, for example by recruiting particular cofactors to the transcription apparatus. Thus far, little is known about the tissue distribution of the other three MiTF/TFE family members TFE3, TFEB and TFEC.
In this study, we have determined the relative expression levels of MiTF, TFE3, TFEB and TFEC in a broad range of human tissues, and identified transcript variants. The ratio of expression of the four MiTF/TFE family members was found to be unique for each tissue. Furthermore, similar to MiTF, we found various splice forms of TFEB and TFEC with alternative first exons and very restricted expression patterns, suggesting regulation through alternative promoter usage.

**MATERIALS AND METHODS**

**cDNA synthesis and RT–PCR**

A panel of total RNA pools from 20 human tissues was obtained from BD Biosciences Clontech (Palo Alto, CA). Reverse transcription was performed using 2 μg of total RNA and Superscript II (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. RT reactions were performed using random hexamers, except for the experiment dealing with alternative intron 7 splicing of TFEC transcripts, for which oligo-dT priming was used to minimize contamination with incompletely spliced nuclear mRNAs. Primers used for PCR were TFEB-1Af (5′-CAAC-AAGGGAAGGTGACATGAAAAGG-3′), TFEB-1Bf (5′-CG-GACAGATTGACCTTCAGGAG-3′), TFEB-1C1f (5′-AT-GCCTAGAGGAGGTTCGGAG-3′), TFEB-1C2f (5′-GA-GAGGAGAGATGAAAACACACC-3′), TFEB-1Df (5′-TTAGACTGCTGCTGGGAAATGCGAG-3′), TFEB-1Ef (5′-TTGAGGTCCTCCGCTGTAACGTGAGG-3′), TFEB-1Ff (5′-CCTCTCTCAGGGGAGCTGCC-3′), TFEB-1Gf (5′-GG-TGTGAGCTCAGGAGACCTTGAGAG-3′), TFEC-1Af (5′-ACTTGTCCAGCAAGACCCACA-3′), TFEC-1Bf (5′-AAATCCAATTCGTCAGCCATGTGGAG-3′), TFEC-1Cf (5′-GAG-AAAACCATGTTATTGAGGAGG-3′), TFEC-1Df (5′-TTGT-GAGCCTATTGATGACTTCC-3′), TFEC-1Ef (5′-TTTCAATGAGGTTGTGG-3′), TFEC-1Ff (5′-CTTCTTGGTCTCCTTCTACATTGCAGC-3′) and TFEC-8r (5′-TCTTTGTTCTCCTTCTACATTGCAGC-3′).

**Real-time quantitative RT–PCR**

Real-time RT–PCR on MiTF/TFE cDNAs was performed on a TaqMan ABI 7700 Sequence Detection System (PE Applied Biosystems) using heat-activated TaqDNA polymerase (Amplitaq Gold; PE Applied Biosystems) as previously described (18). The specificity of the primer/probe sets for each of the four MiTF/TFE members relative to the other three was confirmed using different dilutions of DNA constructs containing the bHLH-LZ-encoding regions of TFE3, TFEB, TFEC or MiTF. The four primer/probe sets recognized their specific MiTF/TFE templates with almost identical efficiencies. For quantitative analysis of the data, the expression levels were normalized to those of endogenous GAPDH (with use of standard TaqMan human GAPDH control reagents; PE Applied Biosystems) using the ΔΔC_{T} technique (29).

**Rapid amplification of 5′-cDNA ends (5′-RACE)**

The 5′-RACE System (Invitrogen) was used according to the manufacturer’s instructions. A TFEB gene-specific primer (5′-ATTTCAGGATTGATGAGC-3′) was used for TFEB cDNA synthesis. The TFEB-specific primers (located in exon 2) were TFEB 5′R442 (5′-TATTGATGGCGGGTGTTGC-3′) and TFEB 5′R376 (5′-TTAATTGCAAGACCTGGTGGC-3′), of which the latter was used for the hemi-nested reaction. For the TFEC 5′-RACE we used the TFEC gene-specific primer (5′-TTTCTAAATGGCTGGTTGG-3′) for cDNA synthesis and primers TFEC 5′R547 (5′-ATTGGTAA-GCTACTTGAGCAAGGACAC-3′) and TFEC 5′R494 (5′-ATTTCCTTGTCGCGCTATACAATCC-3′) for the PCR. The PCR profile was composed of five cycles of 30 s at 94°C, 1 min at 52°C, 30 s at 72°C; five cycles of 30 s at 94°C, 1 min at 52°C, 30 s at 72°C; 25 cycles of 30 s at 94°C, 1 min at 55°C, 30 s at 72°C. PCR products were separated on agarose gels, purified, subcloned into the pGemT vector (Promega, Leiden, The Netherlands), and subsequently sequenced using a Ready Reaction Dye Terminator Cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and an ABI 3700 automated sequencer (PE Applied Biosystems).

**Transfection experiments and western blotting**

Full-length RT–PCR products corresponding to the wild-type TFEB and TFEC transcripts were cloned into the eukaryotic expression vector pSG8-VSV (30). The TFEC cDNAs were cloned in-frame in front of a VSV-tag. Transfection and western blotting was performed as previously described (18). For immunodetection of TFEB, an affinity-purified rabbit polyclonal anti-TFEB-N antiserum was used (18). The VSV-tagged TFEC was detected using the mouse anti-VSV monoclonal antibody PS4D. Immunostaining was performed using chemiluminescence.

**RESULTS**

The MiTF/TFE transcription factor family shows a specific tissue distribution

*In vitro*, TFE3, TFEB, TFE and MiTF form homo- and heterodimers in all possible combinations to bind DNA (1). Based on this observation, it can be hypothesized that differential tissue-specific dimerization may determine the repertoire of target genes that are expressed. In order to analyze the relative expression levels of the MiTF/TFE members in different tissues, we performed quantitative real-time RT–PCR analysis on a panel of 20 human tissues. We established primer sets located within the bHLH-encoding regions, capable of detecting all MiTF/TFE isoforms. As shown in Figure 1A, the expression ratios of the four MiTF/TFE members showed large variations between tissues and none of the members appeared to be co-regulated. In Figure 1B, the same set of data is represented as absolute levels, which allows comparison of the MiTF/TFE expression levels between tissues. Although each of the four members could be detected in all tissues examined, TFEC and MiTF showed more variation in expression between tissues (Fig. 1B). Highest expression levels of both TFE3 and TFEB were found in placenta and lung, but their expression levels differed in adrenal gland (relatively high for TFE3), and prostate and spleen (higher levels of TFEB). MiTF was most prominent in the uterus. The TFEC gene was expressed at lower levels compared to the other three members, and was prominent in spleen, kidney, bone marrow and small intestine. These data
indicate that expression of each of the four members must be regulated in a tissue-specific manner.

The TFEB gene contains alternative 5'-non-coding exons

The temporal and spatial expression of the human MiTF gene is controlled by the differential usage of at least six distinct promoters, driving the expression of transcripts with alternative first exons (24–26, 28). We investigated whether a similar mechanism might act in the other MiTF/TFE members. The TFEB gene, which resides on chromosome 6p21, is composed of nine exons, with a postulated initiation ATG preceded by a perfect ribosomal binding ('Kozak') sequence in exon 2 (18). The numerous TFEB-encoding ESTs that are present in the nucleotide sequence databases reveal the presence of at least three frequently occurring alternative 5′ exons (GenBank Accession nos M33782, NM007162 and AK095061, respectively). To validate whether more TFEB transcripts might exist that differ in their 5'-end, we performed 5'-RACE analysis on cDNA pools of various tissues, using TFEB-specific primers located in exon 2. Using this approach, we were able to identify TFEB transcripts containing seven distinct alternative 5′ exons, of which five were novel, named exons 1a to 1g1 (Table 1 and Fig. 2). The resulting TFEB transcripts were designated TFEB-A to TFEB-G, respectively. The TFEB-B (31) and TFEB-C transcripts were found in liver, kidney, spleen and brain, whereas the remaining five TFEB transcripts were found in only one of the tissues examined (Table 1). In addition, we observed three alternative splicing events in the 5′ region of the TFEB-G transcripts, generating transcripts containing either 1g1 alone, 1g1/1g2 or the complete exon 1g at their 5′-ends (Fig. 2B and Table 1). Similarly, we found alternative TFEB-E splice variants in the brain cDNA pool which contained exon 1c2 and, in some cases, the entire sequence from 1e to 1c2 (Fig. 2B). This latter longest TFEB-E variant corresponds to the previously identified full-length TFEB transcript AK095061. Another 5′-RACE product, obtained from skeletal muscle cDNA, appeared to be an alternatively spliced variant of TFEC-C, lacking exon 1c2 (Fig. 2B). Of all alternative 5′ exons, only exon 1a contains a putative in-frame ATG codon which, if active, would result in 14 extra N-terminal amino acids in TFEB-A. Therefore, most alternative 5′ exons are non-coding. Together, we conclude from these data that the TFEB gene contains multiple alternative first exons.

**Differential tissue distribution of alternative TFEB transcripts**

To analyze the relative expression of the above TFEB variants in human tissues, we performed RT–PCR analysis on the human tissue panel using alternative exon 1-specific primers for the various transcripts. Six TFEB transcripts showed restricted and clearly different tissue distribution patterns (Fig. 2C). Only TFEB-D could not be amplified in any of the tissues, possibly due to very low expression levels. Despite the fact that the splice acceptor site of exon 1d perfectly matches the consensus criteria (Table 1), the TFEB-D transcript was found only once in our 5′-RACE analysis, and its existence thus still needs to be confirmed. TFEB-B and TFEB-C showed the broadest tissue distribution, with TFEB-C being detectable in all tissues but liver. Interestingly, TFEB-A was almost exclusively expressed in placenta, with only minor levels of expression in kidney, lung and prostate. Similarly, the TFEB-E and TFEB-G transcripts appeared to be brain-specific, whereas TFEB-F was highest in spleen (Fig. 2C).

The multiple RT–PCR products observed in the TFEB-E and TFEB-G panels were isolated and sequenced, and appeared to correspond to the splice variants identified with 5′-RACE analysis (Fig. 2B). Based on the intensities of these products in the two panels, these splice variants appear to be expressed in a tissue-specific manner. Despite their differen-

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tial tissue distribution, none of these variants contain upstream ATG start codons, indicating that they all encode TFEB proteins with identical N-termini. Variations in the 5'-UTR sequences might, however, influence the efficiency of translation, as has been described for the cyclin-dependent kinase inhibitor p18INK4c and the nitric oxide synthase gene NOS1 (32,33). We conclude that alternative transcripts may control the expression of TFEB in a tissue-specific manner.

Differential tissue distribution of TFEC variants

In the TFEC gene (Fig. 3), at least two alternative transcription start sites appear to be present, resulting in transcripts here referred to as TFEC-A and TFEC-B (GenBank Accession nos BM807021 and NM012252, respectively). Exon 2 is shared by the two TFEC variants, and contains the ATG start codon. The TFEC-A variant has not been described before, and contains three exons preceding exon 2. Two additional in-frame ATG codons are present in exon 1a3 (Fig. 3A). Using 5'-RACE analysis on human kidney cDNA, we identified a third TFEC transcript, which initiates in an alternative 5' exon positioned between exons 3 and 4 (exon 1c; Fig. 3A). RT–PCR analysis using a reverse primer in exon 1c in combination with a forward primer in any of the upstream exons never resulted in a PCR product, indicating that exon 1c is indeed the first exon of this transcript. TFEC-C thus lacks the protein-coding exons 2 and 3. In-frame ATG start codons were present in both exon

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**Figure 2.** (A) The genomic organization of the human TFEB gene. The ATG start codon in exon 2 and TAG stop codon in exon 9 are indicated. Dotted lines depict alternative splicing. A 1600-bp CpG island colocalizes with exons 1b, 1c1 and part of exon 1d. (B) Alternative splice events in the 5'-regions of TFEB-C, TFEB-E and TFEB-G. Dotted lines depict alternative splicing. The largest of three TFEB-G derived products contained the complete exon 1g, including 1g1, 1g2 and the intermediate 'intronic' sequence. Accession numbers of the nine novel TFEB transcript fragments are AJ608786–AJ608794. (C) Tissue distribution of the human TFEB variants determined by RT–PCR analysis on 20 human tissues.
Subcloning of the TFEC-C cDNA in front of an in-frame VSV-tag, and subsequent transfection into COS-1 cells, revealed that the transcript indeed encodes a protein of the predicted size (~33 kDa; Fig. 3B). A TFEC-C transcript was also found in the mouse (Genbank Accession no. BB843583), and appeared to be highly conserved at both the nucleotide (83%) and amino acid (86%) level.

Similar to TFEB, the tissue distributions of TFEC transcripts was analyzed by RT±PCR (Fig. 3C). TFEC-A was predominantly expressed in testis, thymus, trachea, colon and prostate. TFEC-B showed a broader tissue distribution, and appeared to be absent only from heart and liver, whereas the newly identified TFEC-C transcript was exclusively expressed in kidney and small intestine.

Together, these findings suggest that next to the MiTF gene, the TFEB and TFEC genes also express transcripts with alternative 5’ exons that show restricted and clearly distinct tissue distributions.

Unlike MiTF, TFEB and TFEC, there are no TFE3 transcripts known that would indicate the presence of alternative first exons. Furthermore, RT–PCR analysis on the human tissue panel, using a primer set that included the first exon and a primer set located within the bHLH domain, gave identical tissue distributions (data not shown). Therefore, we assume that expression of the TFE3 gene is regulated by a single promoter.

**Alternative splicing of exons encoding functional domains**

RT–PCRs with 5’ exon-specific primers generated multiple bands in all cases (Fig. 3C), which indicates that from each transcription start site various alternative transcripts can be generated. To reveal their identity, we have isolated and sequenced these different products. The largest RT–PCR product in the TFEC-A panel represents a transcript containing all protein-coding exons, whereas the lower band was found to lack the N-terminal acidic activation domain (AAD)-encoding exon 3 and the basic region-encoding exon 5 (Δ5).

In testis, the tissue that showed the highest expression of TFEC-A, two minor additional products could be isolated as well, which were previously presented as TFEC-L (long) and TFEC-S (short), respectively (34). Apparently, these two
forms are mostly co-expressed, although the ratio in each of the tissues examined was different. Finally, we also detected a weakly expressed, smaller variant of TFEC-C, which differed from the major form in that it again lacked the basic region-
encoding exon 5 (Δ5).

Apart from exon 3 and exon 5, we found indications for another frequently occurring alternative splicing event in the TFEC gene. This alternative splicing event involves the C-terminus of TFEC, which became apparent when we used a reverse RT-PCR primer positioned in exon 8, the 3'-terminal exon of the gene. The C-terminal region of the protein is highly conserved within the MiTF/TFE family and contains two functional domains, namely a proline-rich activation domain (35), and a serine-rich region that contains an Rsk-1 phosphorylation site that is known to play a role in transactivation and degradation of MiTF (36). In most, if not all, TFEC expressing tissues, transcripts could be detected in which intron 7 is still present. Translation of such a transcript would result in a truncated TFEC protein that lacks the proline-rich activation domain and the serine-rich domain (Fig. 3C, two bottom panels). Although the RT–PCRs were performed under semi-quantitative conditions, it appears that the presence or absence of intron 7 in the TFEC transcripts again varies between tissues.

The expression of alternatively spliced variants of TFEB was less apparent than that of TFEC. The TFEB variants were mainly expressed as full-length transcripts that contained an alternative exon 1 followed by the protein-encoding exons 2 to 9. Nevertheless, we did find alternatively spliced TFEB transcripts in a limited set of tissues lacking exon 3 and exon 4, of which the latter encodes the AAD (data not shown).

In conclusion, we have established MiTF/TFE expression levels and ratios in 20 human tissues. These expression patterns appeared to be unique for each tissue. Furthermore, we identified (novel) alternative 5’ exons in the proximal regions of the TFEB and TFEC genes, and demonstrated that the expression of MiTF/TFE isoforms lacking functional domains is a characteristic for all members of the family, but is predominant for TFEC.

DISCUSSION

We have determined the expression profile of the MiTF/TFE family of bHLH-LZ transcription factors in a large series of human tissues and identified alternative transcripts generated by the respective genes. We found that the ratio in which these four members are expressed differs extensively between the various tissues examined. The tight spatial regulation of MiTF/TFE expression appears to be mediated at least in part by alternative transcription start sites. Next to MiTF (4,21,24–28), both TFEB and TFEC were found to express a variety of transcripts that contain alternative 5’ (non-coding) exons and show very restricted differential tissue distributions. The use of alternative 5’ exons in a tissue-specific manner point towards the existence of alternative promoters in both genes. Indeed, through the characterization of an oligo-capped human cDNA library, the exact position of the transcription start site and thereby the position of a core-promoter region has been identified for the TFEB-E transcript (see http://dbtss.hgc.jp/). Furthermore, three more downstream located exons (1b, 1c1 and 1d) are positioned within a 1600-bp CpG island that is conserved between human and mouse. Such regions are known to colocalize with 60% of the human promoters (37). Although we cannot exclude that some of the TFEB variants we found are the result of alternative transcription start site usage within a single promoter region or alternative splicing in the 5’-UTR, our data strongly suggests the presence of alternative promoters in the TFEB and TFEC genes.

We identified seven alternative 5’ exons in the TFEB gene. Except for TFEB-A, all these 5’ exons appeared to be non-coding, suggesting that the respective transcripts encode the same TFEB protein. Together, the TFEB transcripts drive the ubiquitous expression of TFEB. However, each transcript was represented in a restricted set of tissues, and we found three novel TFEB transcripts that were predominantly expressed in one particular tissue. Of these three TFEB transcripts, TFEB-E and TFEB-G appeared to be specifically abundant in brain, whereas TFEB-A was expressed almost exclusively in placenta. Previous studies in transgenic mice already revealed that TFEB, but not the other MiTF/TFE members, plays an essential role in placental vascularization during the early stages of placental development (5,11). Our finding of a placenta-enriched TFEB variant points towards a similar role in the human placenta. This placental expression of TFEB appears to be controlled by a distinct promoter. A total of three alternative 5’ exons were found to be present in the TFEC gene, of which two (exons 1a and 1c) were novel. The three TFEC transcripts were all expressed in a limited set of tissues, with TFEC-C being exclusively found in kidney and small intestine. Despite this restricted expression of TFEC variants, TFEC was observed in all tissues examined, similar to TFEB. From these data, we conclude that next to the MiTF gene, the genes for TFEB and TFEC are also equipped with different alternative first exons and multiple promoter regions. Such a genomic organization allows a tightly regulated expression of the respective transcription factors in a cell type-specific manner, and may explain the observed tissue-specific ratios of the four MiTF/TFE members. These relative expression levels may affect MiTF/TFE homo- or heterodimerization and, thereby, influence the regulation of downstream target genes (18).

Another important mechanism for functional regulation may be the co-expression of MiTF/TFE members with isoforms lacking particular functional domains. Co-expression of such isoforms appears to occur for all MiTF/TFE proteins, but was most evident for TFEC. Multiple splice variants, lacking exons encoding a conserved mitogen-activated protein kinase (MAPK) phosphorylation site (exon 2), the N-terminal AAD (exon 3), the basic region (exon 5), and/or the C-terminal proline-rich activation domain and serine-rich stretch (exon 8), were co-expressed with TFEC-A, TFEC-B and TFEC-C. These domains are highly conserved in the MiTF/TFE family members and all play a role in the transactivating capacities of the proteins. Therefore, these isoforms may act as weaker transactivators or even repressors, as has been shown for TFE3 (35,38) and MiTF (23,36,39). Furthermore, splice variants lacking the basic region have been described for MiTF as well (23), and may act as negative regulators by forming non-DNA-binding homo- or heterodimers similar to what has been proposed for the HLH Id proteins (40,41). The N-terminally truncated TFEC-C isoform appears to be
expressed from an internal promoter, and also lacks the MAPK phosphorylation site and the AAD. Remarkably, this isoform was exclusively expressed in kidney and small intestine, which might indicate that it is involved in the regulation of processes that are shared by these two tissues, like for example (re)absorption of ions and/or organic compounds. The co-expression of these different TFEC isoforms may allow fine regulation of MTF/TFE activities, and may be crucial for the proper spatio-temporal functioning of these transcription factors. Other well-characterized examples of genes that produce multiple isoforms with a regulatory role are p63 and p73, two members of the p53 gene family. Both genes produce splice variants as well as N-terminally truncated forms that originate from internal promoters (42,43). These isoforms act as dominant-negative promoters (42,43). These isoforms may play a prominent role in such regulatory mechanisms.

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