Published in final edited form as:

Brain Res Gene Expr Patterns. 2002 January ; 1(2): 115–121.

Expression of the bHLH transcription factor *Tcf 12* (ME1) gene is linked to the expansion of precursor cell populations during neurogenesis^{*}

M. Uittenbogaard and A. Chiaramello^{*}

Department of Anatomy and Cell Biology, Program of Neuroscience, George Washington University Medical Center, 2300 I Street N.W., Washington, DC 20037, USA

Abstract

In this study, we focused on the potential function of the murine gene *Tcf 12* (also known as ME1 or HEB) encoding the bHLH E-protein ME1 during brain development. An exencephaly phenotype of low penetrance has consistently been observed in both *Tcf 12* null mice and *Tcf 12*^{dm} homozygous mice. Thus, to address the possible underlying mechanism of the *Tcf 12* gene during the early steps of brain development, we performed a detailed analysis of its spatio-temporal expression pattern at distinct steps of gastrulation and neurogenesis. We found that *Tcf 12* transcripts are detected in the embryonic ectoderm prior to neural induction during gastrulation. During neurulation, *Tcf 12* transcripts are evident at high levels in the proliferating neuroepithelium of the neural folds and the cephalic mesenchyme. Thus, *Tcf 12* gene expression coincides with the massive proliferation occurring in the forming neuroepithelium and cephalic mesenchyme during neural tube formation, which is consistent with the exencephaly phenotype of *Tcf 12* null mice. In the developing cortex and spinal cord, *Tcf 12* expression is restricted to the proliferative ventricular zones, indicating that *Tcf 12* expression is down regulated when these neuronal cells undergo their final differentiation. Interestingly, we found that the postnatal *Tcf 12* expression parallels the ongoing adult neurogenesis in the mitotically active subventricular zone. Thus, the timing and location of *Tcf 12* expression combined with this severe neurulation defect support our hypothesis that the *Tcf 12* gene may be involved in the control of proliferating neural stem cells and progenitor cells and that it may be critical to sustain their undifferentiated state during embryonic and adult neurogenesis.

Keywords

bHLH Transcription factor; E-protein; ME1; HEB; Stem cell; Progenitor cell; Gastrulation; Neural tube formation; Proliferation; Postnatal neurogenesis; Subventricular zone

Understanding the molecular events leading to neurogenesis is critical to the advent of cell-replacement therapy in the central nervous system (CNS). To this end, it is essential to understand the underlying mechanisms orchestrating the massive proliferation of stem cells followed by the expansion of the successive waves of progenitor cells destined towards terminal differentiation. In this quest, a great deal of effort has been focused on transcriptional factors regulating the neuronal specification process. Among those found to be critical for this process, the neural specific basic helix–loop–helix (bHLH) transcription factors have been shown to be directly involved in cell lineage restriction, as they contribute to the events of cell

^{*}Published on the World Wide Web on 22 November 2001.

^{*}Corresponding author. Tel.: +1-202-994-2173; fax: +1-202-994-8885. anaec@gwumc.edu (A. Chiaramello).

fate determination and differentiation during neurogenesis (reviewed in [1,14]). However, less attention has been focused on transcription factors that may be promoting the proliferative status. In this regard, one family of transcription factors that may be of interest is the bHLH E-proteins, which are the heterodimeric partners of the neural specific bHLH proteins. The bHLH E-proteins display a broad pattern of expression during mammalian development, and include only three members *Tcf2a* (also known as E2A), *Tcf 12* (also known as ME1, REB, GE1 and HEB in mouse, rat, chicken and human, respectively) and *Tcf 4* (also known as ME2 and E2-2, in mouse and human, respectively). In *Drosophila* as well as in *C. elegans*, only one E-protein has been characterized DA and CeE/DA, respectively [10,13]. All knockout and transheterozygous experiments performed on the three mammalian E-proteins result in postnatal death. An accumulation of detailed analyses of these E-proteins in the immune system suggests that they essentially regulate gene expression by forming homodimeric and heterodimeric complexes between themselves, and that they can substitute each other to a certain degree, suggesting a functional compensation among individual E-protein dimers [3, 4,28]. This is well exemplified by the TCF12 or TCFE2A homodimers in the respective *Tcf 12* and *Tcf2a* knockouts which are capable of compensating for the DNA-binding activity of TCF12–TCFE2A heterodimers, shown to be critical for T-cell development [4]. However, it appears that a critical combined dosage effect of all three proteins is also required to produce the appropriate pro-B-cell population [28]. The regulatory role of the E-proteins has essentially been studied in the context of the developing immune system due to the absence of any immune-specific bHLH regulators (reviewed in [2]). Hence, their role during brain development has not been directly addressed, although their broad expression pattern in the CNS combined with knockout studies suggest that they may regulate brain development. In this report, we focused on the possible function of the *Tcf 12* gene during the early embryonic stages of brain development. In previous studies, *Tcf 12* knockout mice were generated by disrupting the dimerization activity of the TCF12 (HEB) protein, which is the human homologue of the murine TCF12 (ME1) E-protein (92% identity). The phenotype of the *Tcf 12* null mice is characterized by a stunted growth and a high percentage of postnatal death [28]. An analysis of the immune system has shown that the *Tcf 12* null mice also display a slight decrease in pro-B cell numbers, suggesting that the *Tcf 12* gene is not required for the establishment of the B-cell lineage, but is rather involved in the generation of an appropriate pool of pro-B cells [28]. Finally, these *Tcf 12* null mice show a consistent exencephaly phenotype with low penetrance [3,4]. Recently, the function of the *Tcf 12* gene was further investigated by generating a dominant negative *Tcf 12* mutant (*Tcf 12^{dm}*), which carried a point mutation in the basic motif abolishing the DNA-binding activity of both HEB homo- and heterodimers [4]. The *Tcf 12^{dm}* homozygous mice display a severe defect of T-cell development, due to a 5–10-fold reduction in total thymocytes [3,4]. Furthermore, the *Tcf 12^{dm}* homozygous mice are also characterized by an exencephaly phenotype with low penetrance and die shortly after birth, like the *Tcf 12* null mice. Similarly, this low penetrance of the exencephaly phenotype and postnatal death was also observed in *Tcf2a* null mice [4]. It was found that this postnatal lethality could be fully rescued by the addition of two copies of the *Tcf 12* gene under the control of the *Tcf2a* promoter. Furthermore, the levels of TCFE2A protein are increased by two-fold in *Tcf 12* null mice, when compared to wild-type mice. Thus taken together, these observations imply that E-proteins may function during the early steps of brain development in a manner that parallels that associated with the immune system. Hence, the low penetrance of the exencephaly phenotype may be a reflection of functional compensation by other E-proteins. Therefore, these studies suggest that the *Tcf 12* gene may play a critical role in the complex orchestration of neural tube formation and brain development in conjunction with other E-proteins.

Previous studies on the embryonic expression of the *Tcf 12* gene (Genbank accession no. M97635) failed to examine the early stages of neural development [20]. Thus, in this study we carried out a detailed analysis of the spatio-temporal expression pattern of the *Tcf 12* gene at

distinct steps of neural tube formation and neurogenesis. To this end, we generated a *Tcf 12* antisense 677-base-long probe that excluded the bHLH motif to avoid cross-hybridization with other bHLH proteins. We initiated our analysis of the expression of *Tcf 12* transcripts during gastrulation to address the question whether the *Tcf 12* gene was expressed prior to neural induction. Mouse gastrulation starts at about 6.5 days of development with the formation of the primitive streak at the posterior side of the egg cylinder [24]. During this process, a proportion of cells within the epiblast layer migrate towards and through the primitive streak to form the intra-embryonic mesoderm located between the overlying ectoderm and the subjacent embryonic endoderm (reviewed in [5]). Thus, gastrulation has been divided into three stages: early-streak stage (onset of gastrulation), mid-streak stage and late-streak stage. Fig. 1 shows the distribution of the *Tcf 12* transcripts at mid and late streak-stages of gastrulation (E7.0 and E7.5, respectively). In mid-streak gastrulating embryo (E7.0), high levels of *Tcf 12* transcripts were detected in the embryonic ectoderm and the presumptive neuroectoderm in future headfold region (Fig. 1A–D). No signal was detected by a sense probe (data not shown). *Tcf 12* expression was also found in the embryonic mesoderm in the vicinity of the primitive streak, as well as in the notochord plate (Fig. 1A and B). Finally, *Tcf 12* hybridization signal was visualized in several extra-embryonic structures, such as the posterior amniotic fold, amnion and chorion. No *Tcf 12* signal was detected in the decidua. In late-streak gastrulating embryo (E7.5), intense *Tcf 12* expression was observed in the embryonic ectoderm located in the elevated neural folds (Fig. 1E and F). Furthermore, *Tcf 12* transcripts were evident in the embryonic mesoderm, the future cephalic mesenchyme, underlying the forming neural folds. However, we failed to detect *Tcf 12* transcripts in the primitive streak (Fig. 1E and F). *Tcf 12* transcripts were also detected in the extra-embryonic mesoderm tissue of the allantois and the yolk sac. Thus, these results reveal that *Tcf 12* expression is detected in the embryonic ectoderm prior to the onset of neuroectoderm differentiation and is sustained at high levels during neural induction.

During neurulation (E8.0), the *Tcf 12* gene remained highly expressed in the proliferating and differentiating neuroepithelium of the neural folds from the forebrain to hindbrain, as well as in the caudal neural folds (Fig. 2A). High levels of *Tcf 12* mRNA persisted in the cephalic mesenchyme underlying the cranial neural folds and paraxial mesoderm (Fig. 2A). However, *Tcf 12* expression was no longer detected in the extra-embryonic mesoderm and ectoderm layers of the amnion. In the E8.5 turning embryo, *Tcf 12* transcripts remained at high levels in the neuroepithelium, optic eminence, notochord, neural crest-derived head mesenchyme, branchial arches and paraxial mesoderm (Fig. 2B). After rostral neural tube closure, high levels of *Tcf 12* expression were uniformly detected in the forming neuroepithelium, which at this stage is composed of stem cells that continue proliferating to increase their total number without overt differentiation (Fig. 3A). *Tcf 12* expression was also ubiquitous in the caudal neural tube, as the neural folds were about to fuse (Fig. 3A). In addition, *Tcf 12* transcripts were detected in the cephalic mesenchyme, the mesenchyme around the optic vesicle and the otic vesicle as well as the first and second branchial arches (Fig. 3A). Thus, the dynamic pattern of *Tcf 12* expression during gastrulation and neurulation is consistent with the severe neurulation defect observed in *Tcf 12* null mice. These results raise the possibility that the *Tcf 12* gene may control the pool of proliferating precursor cells of the neuroepithelium and cephalic mesenchyme during the early steps of neurogenesis to ensure neural tube closure. It has been demonstrated that abnormal proliferation in both the neuroepithelium and head mesenchyme causes neural tube defects [23]. Two other bHLH proteins, *Hes1* and *Twist*, which are co-expressed with *Tcf 12*, have also been shown to be essential regulators for neural tube closure, as demonstrated by knockout mice [7,12]. The expression of the *Hes1* repressor prevents premature neurogenesis and thus maintains a sufficient pool of dividing neuronal progenitor cells that is critical for neural tube closure [12]. During neurulation, *Twist* promotes the proliferation of the head mesenchyme, which appears to provide a driving force to shape and bend the neural folds [7].

The notion that the *Tcf 12* gene may play a pivotal role in neural precursor proliferation can be extended to more advanced stages of embryonic CNS development. At E12.0, *Tcf 12* transcripts exhibited a gradient of expression in the ventricular zone along the dorsoventral axis of the developing spinal cord (Fig. 3B). *Tcf 12* expression was mainly restricted in the dorsal ventricular zone, where progenitor cells for sensory neurons and interneurons keep proliferating. In contrast, *Tcf 12* expression drastically decreased in the ventral portion of the developing spinal cord, as the progenitor cells for motor neurons ceased to proliferate. This absence implies that *Tcf 12* expression is downregulated when cells undergo their final differentiation. In the developing brain (E12.5), *Tcf 12* expression was abundant in the neuroepithelial wall of the telencephalic ventricle, midbrain and the fourth ventricle (Fig. 4A). No signal was detected with the sense probe (Fig. 4B). It is worth noting that *Tcf 12* transcripts were also detected in the progenitor cells of the cerebellar primordium. In addition, *Tcf 12* remained expressed at high levels in the cephalic mesenchyme. At E15.5, like in the spinal cord, *Tcf 12* expression was restricted to the proliferating ventricular zone of the developing cortex and was excluded from the intermediate zone and the cortical plate (Fig. 4B). Finally, *Tcf 12* expression was sustained in several cranial neural crest derivatives, such as the whisker follicles and the mesenchyme surrounding the nasal sinuses and teeth (Fig. 4B). Thus, the spatio-temporal pattern of *Tcf 12* expression correlates with the expansion of the pool of progenitor cells and the early steps of neuronal differentiation prior to cell cycle withdrawal in the developing CNS.

We have previously shown that postnatal expression of the *Tcf 12* gene is detected in the dentate gyrus of the hippocampus, a region of active neurogenesis in the adult brain [26]. Thus, in this study we extended our postnatal spatio-temporal analysis of *Tcf 12* expression to another area of the brain associated with mitotic activity, the postnatal subventricular zone (SVZ). This zone is a remnant of the embryonic ventricular zone that persists as a thin layer of undifferentiated cells throughout adulthood [15–17]. This proliferative zone contains a heterogeneous population of cells, such as stem cells, mitotically active precursor cells and postmitotic neurons [11,18,19,21]. *Tcf 12* transcripts were detected at high levels in the SVZ during the first 2 postnatal weeks (Fig. 5A and B), a period of active proliferation within this region. *Tcf 12* expression is particularly high in the anterior part of the SVZ, where a majority of olfactory interneurons are generated, and along the rostral migratory pathway to the olfactory bulb, which contains a subpopulation of proliferating cells that will differentiate into granule and periglomerular neurons (Fig. 5A and B). This pattern of expression persisted in adult brain, albeit at lower levels (Fig. 5C). Taken together, these results support the notion that the *Tcf 12* gene may also be involved in the maintenance of the postnatal pool of adult progenitor and stem cells located in the SVZ.

In conclusion, the timing and location of *Tcf 12* expression combined with the exencephaly phenotype observed in *Tcf 12* null mice and *Tcf 12*^{dm} homozygous mice supports our hypothesis that the *Tcf 12* gene may be involved in the control of proliferating neural stem cells and progenitor cells and that it may also be critical to sustain their undifferentiated state during neurogenesis. Consistent with this hypothesis is the recent observation that over-expression of the TCFE2A E-protein promotes the cell cycle proliferation of the 697 B-cell line [27]. Furthermore, in *Drosophila* the absence of the E-protein daughterless causes defects in proliferation, accompanied by a decreased cyclin B expression in cells of the imaginal disk [6]. Finally, this hypothesis is supported by our previous complementary studies showing that overexpression of the TCF12 protein in NGF-treated PC12 cells leads to the repression of the GAP-43 and p75 low-affinity neurotrophin receptor genes, two neuronal-specific genes associated with terminally differentiated neurons [8,9]. The fact that the *Tcf 12* and *Tcfe2a* genes display similar patterns of expression during the embryonic and postnatal proliferative stages of brain development is in agreement with the exencephaly phenotype obtained with both *Tcf 12* null mice and *Tcfe2a* null mice. *Tcfe2a* transcripts are mainly detected in the

embryonic ventricular zone of the developing cortex as well as in the adult SVZ [22]. In contrast, the expression pattern of the *Tcf4* gene does not appear to be limited to the proliferative zones of the developing embryonic and postnatal brain, as it is also expressed in mature postmitotic neurons located in distinct layers of the cortical plate [25]. Therefore, the low penetrance of the exencephaly phenotype may be a reflection of the functional compensation between the E-proteins. Collectively, these studies tentatively lead us to the notion that E-proteins may regulate proliferation of neural progenitor cells in a manner analogous to the immune system. Hence, gene expression during the initial steps of neurogenesis may in part be regulated by a combination of homodimeric and heterodimeric complexes between the three E-proteins. However, E-proteins may also be engaged in heterodimeric complexes with determination factors such as neurogenins and *mash-1* to control neural fate decision. Thus, it would be of interest to unravel the dynamic interplay between TCF12 and the other E-proteins in regulating the transcriptional pathways promoting proliferation during the early stages of neurogenesis. Further studies to elucidate the molecular mechanisms will increase our understanding of TCF12 function during normal and neoplastic proliferation.

2. Materials and methods

2.1. Recombinant plasmids

The full-length cDNA encoding the *Tcf12* gene (Genbank Accession no. M97635) was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) as described in Ref. [24]. The 677-base-pair long *EcoRI*–*Bam*HI fragment corresponding to the nucleotides 990–1667 was subcloned into the pBluescript SK vector and the resulting plasmid was called pBS/ME1.

2.2. In situ hybridization

Embryos and postnatal brains were generated by mating C57BL/6 mice, dissected, and immediately frozen on dry ice. Sections (8 μ m thick) were cut at different stages of the mouse developing nervous system and treated as described [26]. The pBS/ME1 plasmid was linearized with either the *Eco*RI enzyme or *Bam*HI enzyme to generate antisense and sense probes, respectively. Single-stranded sense and antisense mRNA probes (677 bp) were transcribed in vitro using 100 μ Ci [35 S]-UTP (ICN) and T7 or T3 RNA polymerase, respectively. The antisense and sense probes were then diluted to approximately 5×10^4 cpm/ μ l in a buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 10 mM NaPO₄ (pH 6.8), 5 mM EDTA, 1 $\times$ Denhardt's, 10% dextran sulphate, 10 mM DTT and 1 mg/ml tRNA. Hybridization was performed as described in Ref. [26]. The slides were dipped in Kodak NTB-2 emulsion and developed 2 weeks later. They were analyzed by light microscopy with brightfield/darkfield objectives after Giemsa staining.

Acknowledgments

This work was supported by NSF grant (IBN-9510658) and NIH grant (NS R01-41391) to AC.

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Fig. 1.

Tcf 12 transcripts are evident in the embryonic ectoderm prior to the onset of neuroectoderm differentiation and are sustained at high levels during neural induction. Sagittal sections of E7.0 (A–D) and transverse sections of E7.5 (E and F) were hybridized with ^{35}S -labeled *Tcf12* antisense and sense probes. For each stage, brightfield (right panels) and corresponding darkfield (left panels) obtained with the *Tcf12* antisense probe are shown. In mid-streak gastrulation E7.0 embryos, *Tcf 12* transcripts are detected in the embryonic ectoderm, the presumptive neuroectoderm in future headfold region, the notochord plate, and the embryonic mesoderm. In late-gastrulating E7.5 embryo, *Tcf 12* expression is prominent in the forming neural folds and the embryonic mesoderm, the future cephalic mesenchyme. *Tcf 12* expression

is not detected in the primitive streak. The following structures are indicated: al, allantois; am, amnion; ch, chorion; d, decidua; ee, embryonic ectoderm; em, embryonic mesoderm; ne, presumptive neuroectoderm; nf, neural folds; paf, posterior amniotic fold; ps, primitive streak; ys, yolk sac.

Fig. 2.

Tcf12 transcripts are detected in the forming neuroepithelium and cephalic mesenchyme during neurulation. Transverse sections of E8.0 (A and B) and E8.5 (C and D) were hybridized with ³⁵S-labeled *Tcf12* antisense and sense probes. For each stage, brightfield (right panels) and darkfield (left panels) pictures obtained with the *Tcf12* antisense probe are shown. At E8.0, *Tcf12* expression is abundant in the cephalic mesenchyme and the neuroepithelium of the neural folds. At E8.5, *Tcf12* expression is also detected in the forming branchial arch, notochord, and the optic eminence. The following structures are indicated: ba, branchial arch; cm, cephalic mesenchyme; d, decidua; fb, forebrain; hb nf, hindbrain neural folds; nf, neural folds; no, notochord; pm, paraxial mesoderm.

Fig. 3.

Tcf12 transcripts remain expressed in neuroepithelium stem cells after neural tube closure and in proliferative progenitor cells of the developing spinal cord. Transverse sections of embryonic day E9.0 (A) and E12 (B) were hybridized with ^{35}S -labeled *Tcf12* antisense and sense probes. For each stage, only darkfield pictures obtained with the *Tcf12* antisense probe are shown. At E9.0, *Tcf12* expression remains abundant in neuroepithelial stem cells after neural tube closure. This section also illustrates the expression of *Tcf12* transcripts in the caudal neural tube as the neural folds just fused. *Tcf12* is also expressed in the cephalic mesenchyme, the mesenchyme around the optic vesicle and the otic vesicle, and the branchial arches. At E12, *Tcf12* expression is detected in proliferating progenitor cells giving rise to sensory neurons and interneurons. The following structures are indicated on the figure: am, amnion; ba, branchial arch; cm, cephalic mesenchyme; fp, floor plate; mb, midbrain; nt, neural tube; op, optic vesicle; ot, otic vesicle; rf, roof plate; vz, ventricular zone.

Fig. 4.

Expression of the *Tcf 12* gene is associated with the expansion of precursor cell populations during brain development. Sagittal sections of embryonic day E12.5 (A and B) and E15.5 (C and D) were hybridized with ^{35}S -labeled *Tcf12* antisense and sense probes, as shown in left and right panels, respectively. *Tcf 12* expression is detected at high levels in the ventricular zone of the telencephalon, midbrain, pons, and medulla oblongata. In the cerebellar anlage, *Tcf 12* is expressed in the rhombic lip cells and the progenitor cells located in the cerebellar primordium. *Tcf 12* transcripts are also detected in the cephalic mesenchyme. At E15.5, *Tcf 12* expression is restricted to the ventricular zone of the developing cerebral cortex. *Tcf 12* expression is sustained in the mesenchyme around the whisker follicles, the nasal sinuses and teeth. The following structure are indicated: cb, cerebellum; mb, midbrain; mes, mesenchyme; nc, nasal cavities; p, pons; t, tongue; tb, tooth bud; tel, telencephalon; vz, ventricular zone.

Fig. 5.

Postnatal expression of the *Tcf 12* gene is observed in mitotically active areas of the brain. Sagittal sections of newborn (A), postnatal day P14 (B), and 3 month-old adult brain (C) were hybridized with ^{35}S -labeled *Tcf12* antisense and sense probes. For each stage, only the darkfield pictures obtained with the *Tcf12* antisense probe are shown. In a newborn brain, *Tcf 12* transcripts are highly detected in subventricular zone, the rostral migratory pathway, and the olfactory bulb. In an adult brain, *Tcf 12* remains expressed at a significant level in the subventricular zone. The following structures are indicated: CA1, Ammons horn; cc, corpus callosum; ctx, cortex; DG, dentate gyrus; ob, olfactory bulb; rmt, rostral migratory tract; svz, subventricular zone.