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T-Brain-1: A Homolog of *Brachyury* Whose Expression Defines Molecularly Distinct Domains within the Cerebral Cortex

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Summary

The mechanisms that regulate regional specification and evolution of the cerebral cortex are obscure. To this end, we have identified and characterized a novel murine and human gene encoding a putative transcription factor related to the *Brachyury* (*T*) gene that is expressed only in postmitotic cells. *T-brain-1* (*Tbr-1*) mRNA is largely restricted to the cerebral cortex, where during embryogenesis it distinguishes domains that we propose may give rise to paleocortex, limbic cortex, and neocortex. *Tbr-1* and *Id-2* expression in the neocortex have discontinuities that define molecularly distinct neocortical areas. *Tbr-1* expression is analyzed in the context of the prosomeric model. Topological maps are proposed for the organization of the dorsal telencephalon.

Introduction

A major challenge in biology is to understand how the components of the forebrain are formed and are then interconnected. As a first step, efforts are underway to determine the organization of the neural plate and the early neural tube. Fate maps of the chick and frog neural plate have already established the location for many of the forebrain primordia (Couly and Douarin, 1988; Eagleson and Harris, 1989; Bortier and Vakaet, 1992). Maps based on the expression of candidate regulatory genes have begun to define the position of boundaries that delimit potential histogenic zones and early axons pathways (Bulfone et al., 1993a; Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Stoykova and Gruss, 1994; Macdonald et al., 1994; Alvarez-Bolado et al., 1995). Available evidence suggests that there are two types of boundaries. One type is perpendicular to the longitudinal axis; these transverse boundaries segregate transverse or neuromeric domains. The other type of boundary is parallel to the longitudinal axis; these boundaries segregate longitudinal domains that extend through multiple transverse domains. The results of our studies have led to the prosomeric model, which proposes that the forebrain has a neuromeric organization (Bulfone et al., 1993a; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Molecular genetic approaches contribute to our understanding of the mechanisms that pattern the embryonic forebrain. The first step is to identify genes that encode candidate regulatory molecules. To this end, we used subtractive hybridization to isolate cDNAs encoded by genes that are preferentially expressed during forebrain development (Rubenstein et al., 1990; Porteus et al., 1992). This approach led to the isolation of a novel homeobox gene named *Tes-1*. Subsequently, *Tes-1* was renamed *Dlx-2* because of its homology to four other members of the murine *Distal-less* gene family (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994). CNS expression of the *Dlx* genes is restricted to the forebrain, where they are transiently expressed during midgestation in the basal telencephalon, ventral thalamus, and hypothalamus (Bulfone et al., 1993a, 1993b; Porteus et al., 1994; Simeone et al., 1994).

There is a rapidly growing list of genes that are expressed in regionally restricted domains of the embryonic forebrain. For instance, there are over 25 homeobox genes expressed in the embryonic forebrain (Rubenstein and Puelles, 1994). These results show that the forebrain is subdivided into molecularly distinct domains before activity-dependent processes can have a patterning role. Though these findings unequivocally demonstrate subdivisions of embryonic diencephalon, hypothalamus, and basal telencephalon, there is very little data that delineates the early subdivisions of the cerebral cortex.

The cerebral cortex is subdivided into several histologically and functionally distinct regions. The clearest subdivisions are the six-layered iso- or neocortex (NC), and the non-six-layered allocorteces (paleocortex, archicortex, and transitional corteces) (Zilles and Wree, 1995). Each of these areas is further compartmentalized. For instance, the NC has sensory, motor, and associational areas. There is very little information that explains how these major subdivisions are formed, though there is increasing evidence for the role of thalamic inputs in patterning subdivisions of the NC (O'Leary et al., 1994). For instance, transplantation of fetal visual cortex into neonatal sensory cortex results in a sensory cortex with many of its normal properties (Schlaggar and O'Leary, 1991). However, these findings do not resolve how thalamic afferents recognize their appropriate target zone within the cortex, and they suggest that there is a molecular map of the embryonic cortex

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that defines the spatial coordinates within the cortical field (Rakic, 1988).

An approach to determining the mechanisms that set up the spatial coordinates that pattern the cortex is to identify molecules whose expression defines discrete cortical domains. In the last several years, a number of genes have been cloned that are expressed in the embryonic cortex. These include homeobox genes (*Otx-1*, *Emx-1*, *Emx-2* [Simeone et al., 1992a, 1992b, 1993], *Brn1*, *Brn2* and *Tst-1* [*SCIP;* He et al., 1989; Wegner et al., 1993], *Prox-1* [Oliver et al., 1993], *Dlx-2* [Porteus et al., 1991], and *Pax-6* [Stoykova and Gruss, 1994]), as well as nonhomeobox transcription factors (*BF-1* [Tao and Lai, 1992], *Id-2* [Neuman et al., 1993; Zhu et al., 1995], and *N-Myc* and *Heir-1* [Ellmeier et al., 1992]) and growth/differentiation factors (*Wnt-7b* [McMahon et al., 1992]). Recently, the discovery of a genetic locus that controls the expression of a *lacZ* transgene in the somatosensory cortex has been reported (Cohen-Tannoudji et al., 1994). This finding implies that development of specific neocortical regions have distinct genetic programs. In addition, workers are searching for genes that control histogenesis of the cerebral cortex by regulating migration to the appropriate cortical layer and differentiation. Several transcription factors (*Tst-1* [*SCIP*], *Otx-1*, and *Id-2*) have been shown to be preferentially expressed in specific cortical laminae (Neuman et al., 1993; Frantz et al., 1994a, 1994b).

In this paper, we describe the isolation of a novel gene named *T-Brain-1* (*Tbr-1* or *Tes-56*), which stands for T-box Brain gene, that was obtained from the same subtractive hybridization that yielded the *Dlx-2* (*Tes-1*) gene. *Tbr-1* is a homolog of *Brachyury* (also known as the *T* locus gene), a transcription factor essential for axial mesoderm development (Herrmann et al., 1990; Schulte-Merker et al., 1992; Kispert and Herrmann, 1993) and to three other newly discovered murine members of the *Brachyury* family named *Tbx-1*, *Tbx-2*, and *Tbx-3* (Bollag et al., 1994). *Tbr-1* is the only known member of this family that is extensively expressed in the vertebrate CNS.

Tbr-1 is expressed in postmitotic cells in the forebrain. Though the onset of its expression is during embryogenesis, it continues to be expressed in the adult brain, preferentially in specific layers of the cerebral cortex. By comparing the expression of *Tbr-1* with the *Id-2* gene, we have identified boundaries of expression within the prenatal and postnatal NC. One of these boundaries is present in the superficial cortical plate at E16.5, a time when thalamic afferents are just beginning to enter the deep cortical plate. In addition, *Tbr-1* is expressed at different levels in distinct bands of cells in the embryonic cortex that we propose may correspond to its paleocortical, limbic, and neocortical subdivisions.

Results

Cloning, Sequence, and Chromosomal Location of *Tbr-1*

To identify genes that are candidates for regulating mouse forebrain development, we performed a subtractive hybridization between cDNA libraries made from RNA expressed in the embryonic day 14.5 (E14.5) telencephalon and the adult telencephalon (Porteus et al., 1992). The subtracted library contains cDNAs encoded by genes that are preferentially expressed in the developing telencephalon. We have analyzed the subtracted cDNA library using DNA sequencing and in situ RNA hybridization in a search for genes with structural motifs that suggest a regulatory function and for regionally restricted patterns of expression. Using this approach, we have identified a novel gene that is expressed primarily in the telencephalic vesicle (see Figures 3–7) that we initially named *Tes-56*.

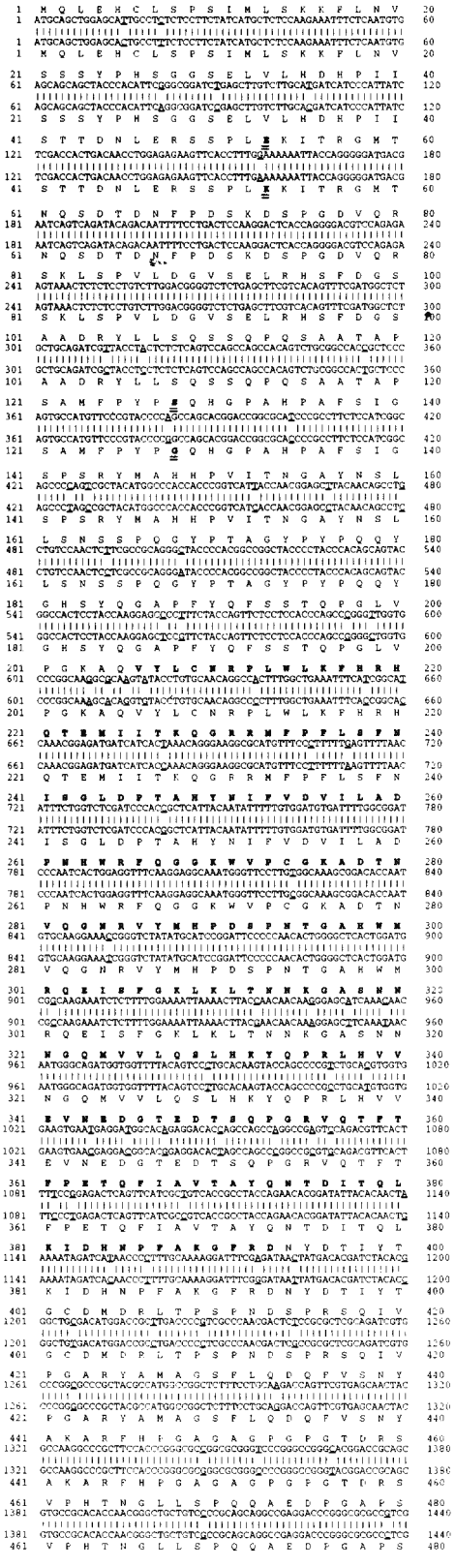
Northern analysis of *Tes-56* revealed a single 3.3 ± 0.4 kb transcript that is about 10-fold more abundant in the E14.5 telencephalon then in the adult telencephalon (data not shown). DNA sequence analysis of a 3.805 kb cDNA clone and genomic DNA showed that *Tes-56* encodes a 681 amino acid protein that has a 188 amino acid domain that is highly homologous to the *Brachyury* or *T* gene (Herrmann et al., 1990; Schulte-Merker et al., 1992; Kispert and Herrmann, 1993). Other homologs of *Brachyury* have also been identified (*Tbx-1*, *Tbx-2*, *Tbx-3*, and *optimotor blind* [*omb*]; Pflugfelder et al., 1992; Bollag et al., 1994). The region of homology between these genes is termed the T-box (Bollag et al., 1994). Because *Tes-56* is related to these genes, we have renamed it *Tbr-1*.

To determine whether any of the known strains of mice might harbor mutations in the *Tbr-1* gene, we determined its chromosomal position. *Tbr-1* is on mouse chromosome 2 approximately 33 cM from the centromere (see Figure 2C). The mutations that have been mapped to this area are *fidget* (Yakovlev et al., 1977), *lethargic* (Herring et al., 1981), and *muscular dystrophy with myositis* (Lane, 1985). Based on the expression pattern of *Tbr-1*, it seems unlikely that the phenotypes described for these mutations are due to mutation of *Tbr-1*.

Murine and Human *Tbr-1* Are Members of the T-Box Family of Transcription Factors

To study the evolution of the *Tbr-1* family, we isolated the human ortholog. We sequenced a human 2.9 kb cDNA and compared it with the murine *Tbr-1* cDNA sequence (Figure 1). The human cDNA encodes a 682 amino acid TBR-1 protein. The mouse cDNA is judged to be missing 8 bp of coding sequence; but, based upon sequencing of the mouse *Tbr-1* gene, it encodes 681 amino acids.

Comparison of the human and mouse amino acid *Tbr-1* sequences reveals colinearity of the proteins, except for an insertion of 1 amino acid (glycine at position 599) and identity at 675 of the 681 amino acids (Figure 1). Within the T-box, there is 100% identity. We then compared the T-box of *Tbr-1* with the T-box from other mouse (*Brachyury*, *Tbx-1*, *Tbx-2*, and *Tbx-3*), Drosophila (*omb*), and C. elegans genes (Figure 2A; Wilson et al., 1994). Outside of the T-box, there are also amino acid identities found between *Tbr-1* and the other T-box genes (data not shown). Homologies within the T-box allowed us to determine a consensus sequence, as well as to determine a dendogram that predicts evolutionary relationships within this new gene family (Figure 2B). These results are addressed in the Discussion.



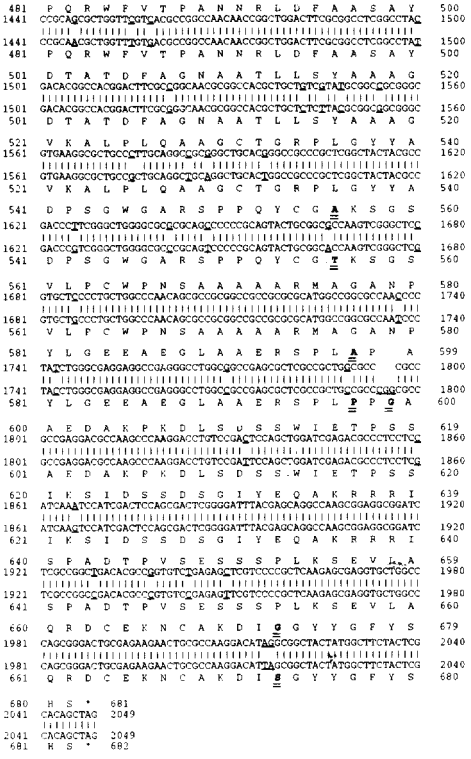


Figure 1. Nucleotide and Deduced Amino Acid Sequence of cDNA Clones Encoding Transcribed Regions of the Mouse and Human *Tbr-1* Genes

The mouse sequence is above the human sequence. Amino acids are shown using the one letter code; amino acid differences between the mouse and human proteins are in double underlined letters. Nucleotide differences are underlined. The T-box is in bold.

Expression of *Tbr-1*

Onset of Expression

Expression of *Tbr-1* was studied using in situ RNA hybridization to section and whole-mount preparations. The terminology used to describe its expression is based on the prosomeric model (see Figure 8; Bulfone et al., 1993a; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). No expression is detected at E7.5, E8.5, or E9.5. Expression was first detected around E10.0, when *Tbr-1* RNA is found in the mantle zone of the telencephalic vesicle (Figure 3a). Initially, expression is found in the rostroventral region; by E10.5 it extends caudodorsally to cover much of the telencephalic vesicle (data not shown). Expressing cells are not homogeneously arranged on the surface of the telencephalon; rather, clusters of cells are connected in a reticular pattern. Expression is excluded from the dorsal midline.

Tbr-1 *Is Expressed in Postmitotic Cells*

Beginning at E10.0, *Tbr-1* is expressed in a single layer of superficial cells in the telencephalon (Figure 3a) and is excluded from the ventricular zone (VZ; data not shown). This layer of cells is known as the preplate in NC; it gives rise to the marginal and subplate zones. We will use the term mantle zone (MZ) to describe the stratum of postmi-

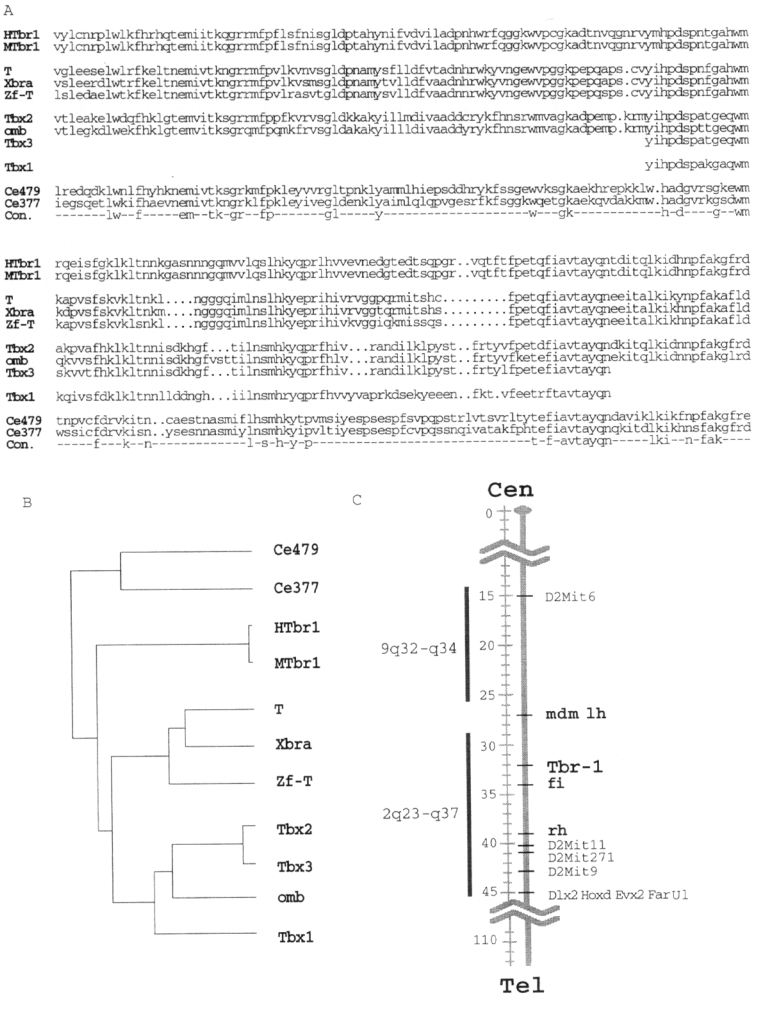


Figure 2. *Tbr-1* Is a Member of the T-Box Gene Family

(A) Comparison of the conserved amino acid regions (T-box) of mouse and human *Tbr-1* (MTbr, HTbr) with the T-gene from mouse (*T*), Xenopus (*Xbra*) and zebrafish (*Zf-T*), mouse *Tbx-1*, *Tbx-2*, and *Tbx-3*, Drosophila *optimotor blind* (*omb*), and two C. elegans genes (*Ce479*, *Ce377*). A concensus sequence of amino acids that are identical in these 11 genes is shown on the bottom line.

(B) Dendogram analysis of T-box genes defines subgroups within this gene family.

(C) Map of mouse chromosome 2, showing the location of Tbr-1. Syntenic regions from the human genome are indicated on the left. The names of mouse loci are listed to the right.

totic cells, since *Tbr-1* is also expressed in telencephalic regions other than the NC that are not known to contain a preplate, subplate, cortical plate, and marginal zone. By E12.5, the MZ can easily be distinguished from the VZ by differential staining with toluidine blue; the MZ cells are paler than VZ cells (Figure 3e). In situ hybridization shows that *Tbr-1* is expressed only in the MZ cells (Figures 3d, 3d′, and 3f). To demonstrate the restriction of *Tbr-1* to postmitotic cells, the most superficial layer of the VZ was labeled with a 1 hr pulse of bromodeoxyuridine (BrdU; Figure 3g). Comparison of BrdU and *Tbr-1* stained adjacent sections confirms that *Tbr-1* expression begins after neuroepithelial cells have left the cell cycle and migrated into the MZ.

Tbr-1 *Expression Is Spatially Restricted in the Forebrain and Is Excluded from* Dlx-2*-Positive Domains*

The expression of *Tbr-1* was compared with the *Dlx-2* homeobox gene at E12.5 in sagittal and coronal sections (Figure 4 and summarized in Figure 8). These genes show complementary expression patterns in the forebrain. Within the telencephalon, *Tbr-1* is expressed in areas that we propose are the primordia of the paleocortex, NC, and archicortex (Figures 4e–4h), as well as in the primordia of the anterior olfactory nucleus and diagonal band nucleus (Figures 4e and 4f). *Tbr-1* is also expressed in the caudal ganglionic eminence (Figure 4h), eminentia thalami (Figures 4e and 4g), and the primordium of the bed nucleus of stria medullaris (Figures 4e and 4h).

Tbr-1 is excluded from most of the ventral region where *Dlx-2* is expressed. There is a sharp boundary between the *Tbr-1*-positive NC and the *Dlx-2*-positive lateral ganglionic eminence (LGE; compare Figures 4e and 4i, 4f and 4j, and 4g and 4k). Nonoverlapping expression is also found more caudally when comparing the *Tbr-1*-positive eminentia thalami and stria medullaris with the *Dlx-2*-positive ventral thalamus, anterior entopeduncular area, and supraoptic paraventricular area (compare Figures 4e and 4i, 4g and 4k, and 4h and 4l). The only region where there is some overlap of expression is in the region of the diagonal band in the rostroventral telencephalon (compare Figures 4e and 4i, 4f and 4j, and Figure 8). There is no *Tbr-1* expression in the chorioidal tela (Figure 4f).

Tbr-1 *Expression Suggests the Fate of Telencephalic Primordia*

The expression pattern of *Tbr-1* changes little throughout development (compare Figures 3–7). This has allowed us provisionally to assign the fates of various *Tbr-1*-positive

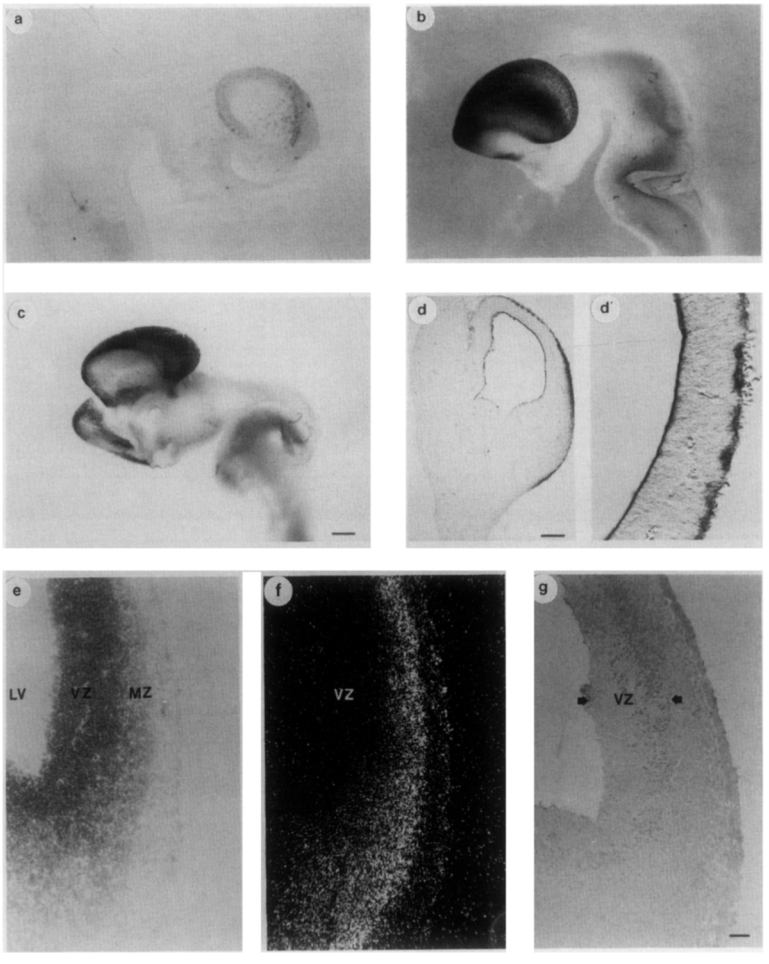


Figure 3. *Tbr-1* Is Expressed in Postmitotic Cells of Regionally Restricted Domains of the Embryonic Cerebral Cortex

(a–d) *Tbr-1* expression using nonradioactive whole-mount in situ RNA hybridization.
(a) A lateral view of the brain at E10.0.
(b and c) Lateral and ventrolateral views of an E12.5 brain.
(d) A section through the forebrain of an E12.5 brain, which illustrates the different level and pattern of *Tbr-1* expression in different regions of the cerebral cortex.
(d') The dorsolateral cortex in (d), showing that *Tbr-1*- positive cells are restricted to the outermost layer and are organized in clusters.
(e–g) *Tbr-1* is expressed in postmitotic cells of the cerebral cortex.
(e) Toluidine blue stain; the locations of the lateral ventricle (LV), ventricular zone (VZ), and mantle zone (MZ) are indicated.
(f) *Tbr-1* expression detected by in situ RNA hybridization.
(g) BrdU incorporation detected using immunohistochemistry. The width of the ventricular zone (VZ) is shown by arrowheads.
Bars, 380 μm (c); 100 μm (d); 50 μm (g). (a) is enlarged 2-fold more than (b–d); (d') is enlarged 4-fold more than (d).

primordia. The paleocortical cortical anlage (Figure 4) gives rise to the anterior olfactory nucleus (see Figures 7b and 7c), accessory olfactory bulb (see Figure 7c), and the olfactory bulb (see Figures 7a, 7b, and 7c) and the piriform cortex (see Figure 7d) and endopiriform nucleus (see Figure 7d). Within the postnatal olfactory bulb, *Tbr-1* is largely found in a single layer that appears to correspond to the mitral cells (see Figures 7a and 7b). The neocortical anlage (Figure 4) gives rise to the NC (see Figures 7a–7d), which exhibits laminar and regional differences in *Tbr-1* expression (see following sections). The archicortical anlage (Figure 4) gives rise to the *Tbr-1*-positive hippocampus (see Figures 7b, 7c, and 7d), subiculum (data not shown), entorhinal cortex (see Figures 7c and 7d), indusium grisium (data not shown), and tenia tecta (data not shown).

Similarly, *Tbr-1* is expressed in subcortical forebrain domains. The region between the LGE, paleocortex, and NC is *Tbr-1* positive (Figure 4) and may form the claustrum (see Figure 7d). The caudal ganglionic eminence (Figure 4) appears to give rise to parts of the amygdala (see Figure 6e and Figure 7d) and perhaps the nucleus of the lateral olfactory tract (see Figure 7d). The basal telencephalic zone labeled DB (see Figures 6g and 6h) gives rise to the vertical and horizontal limbs of the diagonal band (see Figures 7a–7c).

Tbr-1 expression in the forebrain is limited to the telencephalic vesicles except for one small ventral projection through the caudal stalk region called the eminentia thalami (Figures 4e and 4g) and into a caudodorsal domain that may correspond to the primordia of the nucleus of stria medullaris (Figures 4e and 4h) based on continued expression in this region postnatally (see Figure 7c).

Tbr-1 *Expression Shows Regional Differences in the Embryonic Telencephalic Vesicle*

Whole-mount in situ hybridization at E12.5 reveals distinct bands of cells that express different levels of *Tbr-1* in the telencephalic vesicle (see Figures 3b and 3d; see Figure 8). Ventrally, there are two longitudinal stripes of cells that express *Tbr-1* at higher levels. The more dorsal band is wider and extends caudally from the anlage of the olfactory bulb to the caudal pole of the telencephalon. The more ventral band also connects the olfactory bulb to the caudal pole of the telencephalon, thereby forming a continuous belt of strong expression that circumscribes a central area of lower expression. We postulate that this ventral–lateral telencephalic region includes the prospective paleocortical region that is covered by the olfactory tract and the lateral limbic cortex (see Discussion).

Dorsal to the putative paleocortical and lateral limbic anlage is the primordia of the NC, which has a lower level of *Tbr-1* expression. Unlike in the ventrolateral cortex, where

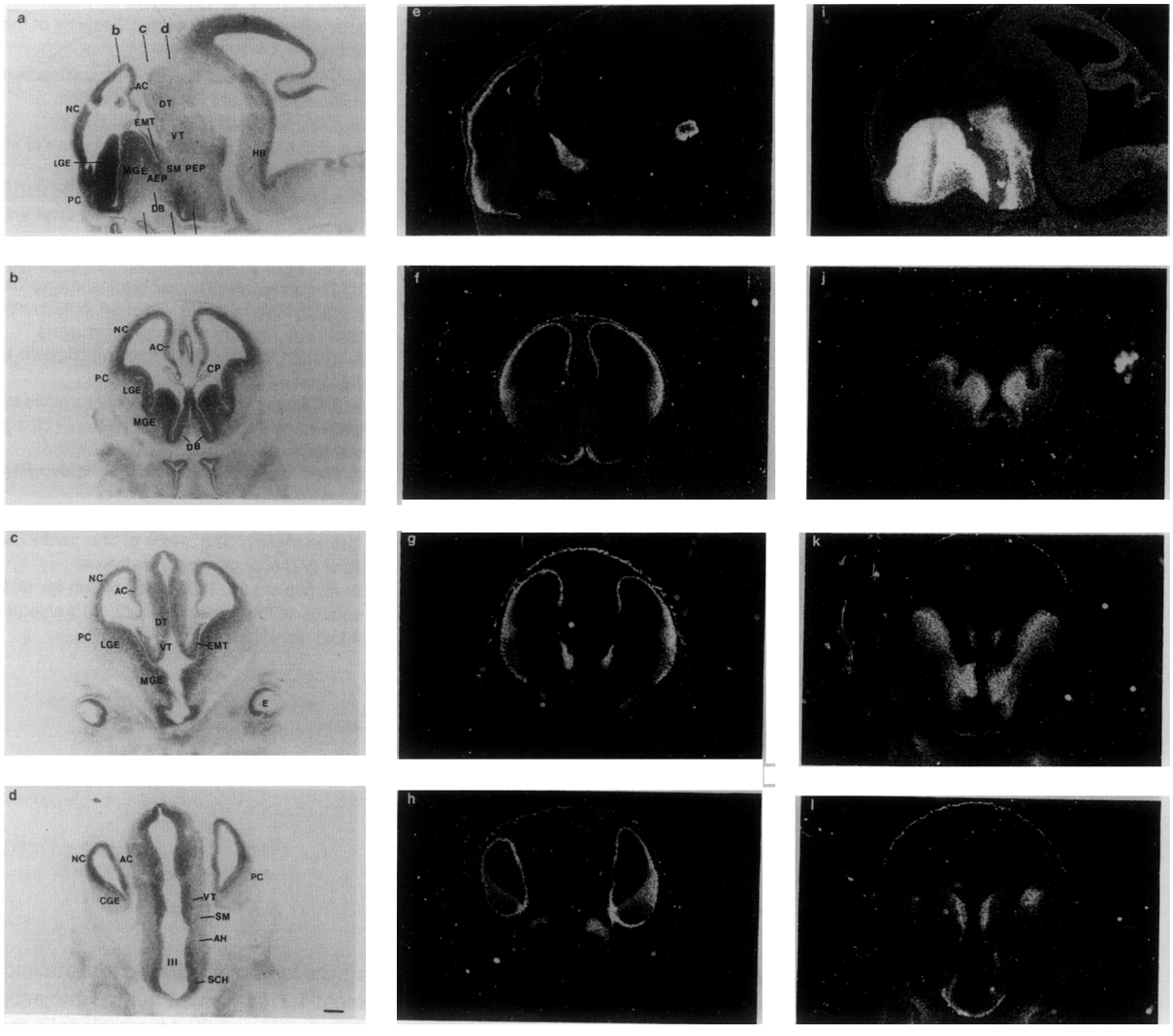


Figure 4. *Tbr-1* and *Dlx-2* Have Complementary Patterns of Expression in the Brain at E12.5 Revealed by In Situ Hybridization

(a–d) Toluidine blue–stained sections; (e–h) expression of *Tbr-1*; (i–l) *Dlx-2* expression. The top tier shows parasagittal sections. The bottom three tiers show coronal sections; the approximate planes of the coronal sections are shown in (a). Bar, 250 μm.

AC, archicortex; AEP, anterior entopeduncular area; AH, anterior hypothalamus; CGE, caudal ganglionic eminence; CP, chorioid plexus; DB, diagonal band; DT, dorsal thalamus; E, eye; EMT, eminentia thalami; HB, hindbrain; III, third ventricle; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NC, neocortex; PC, paleocortex; PEP, posterior entopeduncular area; SCH, suprachiasmatic area; SM, stria medullaris; VT, ventral thalamus.

expression appears to be uniform, expression in the NC has a reticular pattern consisting of "clusters" of *Tbr-1* expressing cells (see Figures 3b and 3d′). These *Tbr-1*-positive clusters are also seen at the outset of *Tbr-1* expression at E10.0 (see Figure 3a).

Restricted Rostrocaudal Expression of Tbr-1 *in the NC*

As noted above, *Tbr-1* expression begins in the preplate of the NC around E10.0. Expression is maintained in postmitotic cells into adulthood. In addition to the discrete bands of expression in the lateral wall of the E12.5 cortex (see Figures 3b and 3d), *Tbr-1* exhibits other discontinuities in its expression by E16.5.

A boundary of *Tbr-1* expression along the rostrocaudal axis of the neocortical plate is first apparent around E16.5 (Figure 5); Tbr-1 is expressed rostral to the boundary. This boundary is found in the superficial zone of the neocortical plate at E16.5, E18.5 (Figure 5 and Figure 6), and PO (data not shown). The position of this boundary, which is marked by open arrows in Figure 5 and Figure 6, shifts caudally in more medial regions of NC. To quantitate the differential expression of *Tbr-1* at E16.5 in the rostral high expression zone (Figure 5c, 1) compared with the caudal low expression zone (Figure 5c, 2), we counted the number of silver grains produced by the autoradiography reaction. The superficial strata of the high expression zone had 18 ± 7 grains/16 μm^2, whereas the superficial strata of the low expression zone had 3 ± 2 grains/16 μm^2. The deep

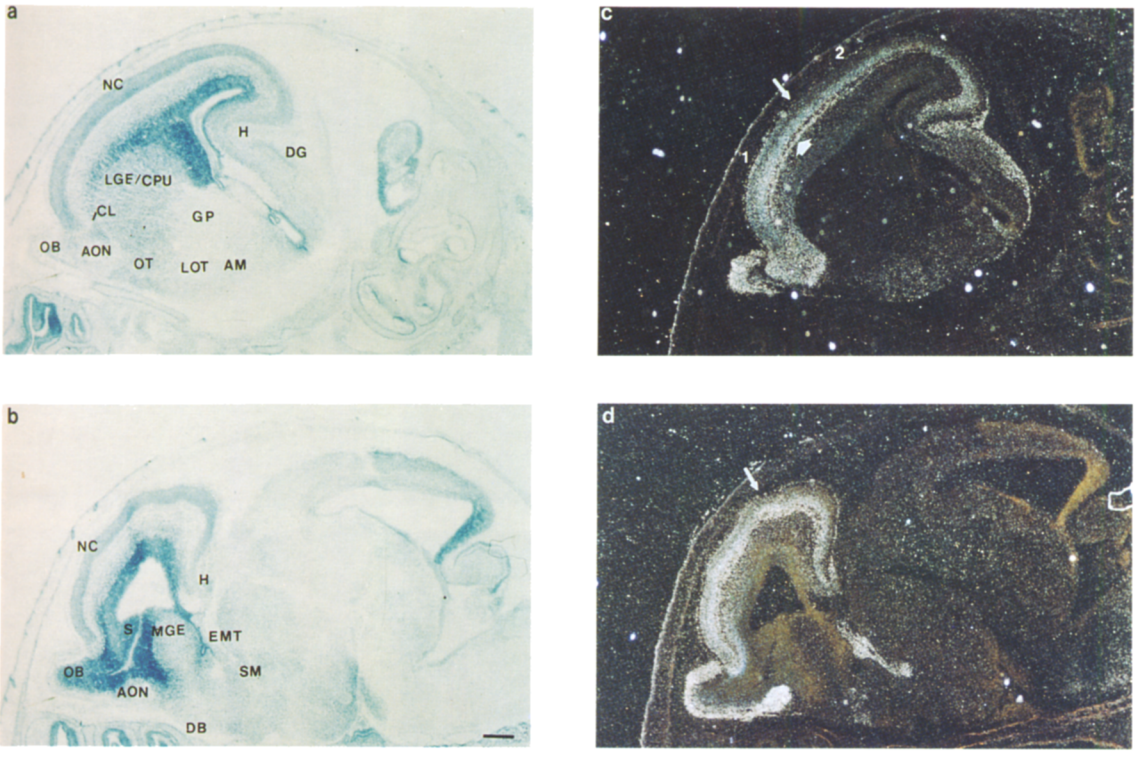


Figure 5. Discontinuity of *Tbr-1* Expression in the Superficial Part of the Cortical Plate at E16.5 Revealed by In Situ Hybridization

(a and b) Toluidine blue–stained sections; (c and d) expression of *Tbr-1*. The thin arrows in panels (c) and (d) show the position where *Tbr-1* expression ends in the superficial part of the cortical plate. Numbers 1 and 2 indicate regions where silver grain density were determined (see text). The wide arrowhead (c) shows *Tbr-1* expression in a layer that probably corresponds to the subplate. Bar, 630 μm.

AM, amygdala; AON, accessory olfactory nucleus; CL, claustrum; CPU, caudoputamen; DB, diagonal bands; DG, dentate gyrus; EMT, eminentia thalami; GP, globus pallidus; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NC, neocortex; OB, olfactory bulb; OT, olfactory tubercle; S, subiculum; SM, stria medullaris.

strata in these two regions did not have significantly different numbers of silver grains (rostral, 20 ± 3 grains/16 μm^2; caudal, 18 ± 6 grains/16 μm^2). Thus, in the superficial cortical plate, *Tbr-1* is expressed about 6-fold less caudally than rostrally. The transition is gradual between these two extremes (Figure 5c, arrow), and takes place over approximately 500 μm.

There is also a discontinuity of expression that approximates the boundary of the hippocampus with the subiculum that is first apparent at E18.5 (Figures 6e–6g). The expression in the subiculum decreases abruptly upon entering the hippocampal primordium (Figure 6g, triangle). Postnatally, there remains an attenuation in *Tbr-1* expression at the boundary of the subiculum with CA1 (see Figures 7b and 7c); however, high levels of hippocampal expression are found beginning at approximately CA3 and continuing into the dentate gyrus (see Figures 7b–7d).

Restricted Laminar Expression of Tbr-1 *in the NC: Comparison with the Expression of* Id-2

Tbr-1 is expressed in specific lamina in the developing and adult cerebral cortex. As already noted, *Tbr-1* expression is limited to the nonproliferative cortical strata. Beginning at E10, it is expressed in the preplate. By E12.5 it is expressed in the cortical plate and intermediate zone. At E16.5 and E18.5, expression is seen in a thin layer of cells between the cortical plate and the intermediate zone, which may be the subplate (Figure 6g, arrowhead). Also, as noted above, there is much less *Tbr-1* expression in the caudal superficial cortical plate at E16.5 (Figures 5c and 5d) and E18.5 (Figures 6f–6h).

Within the postnatal NC, *Tbr-1*expression is found at different levels in distinct layers. To help identify the cortical layers that express *Tbr-1*, we studied the expression of the *Id-2* gene. Previously, it had been shown that *Id-2* is expressed in layers 6, 5, and 2/3 of the NC (Neuman et al., 1993). We confirmed this result (Figures 7e–7h). Comparison of neighboring sections in which *Id-2* and *Tbr-1* expression are alternately stained (Figure 7) provides evidence for the position of the *Tbr-1*-positive laminae, which are described below.

At P7 (Figures 7b, 7c, 7f, and 7g), the interface of the white matter and the NC has a thin strip of *Tbr-1*- and *Id-2*-positive cells that may correspond to the subplate. Above this is layer 6, which expresses both genes. Superficial to layer 6, there is much less *Tbr-1* and *Id-2* expression in a zone that may correspond to a sublamina of layer 5. Just superficial to this negative zone, *Id-2* has very strong expression in a layer that previously had been identified

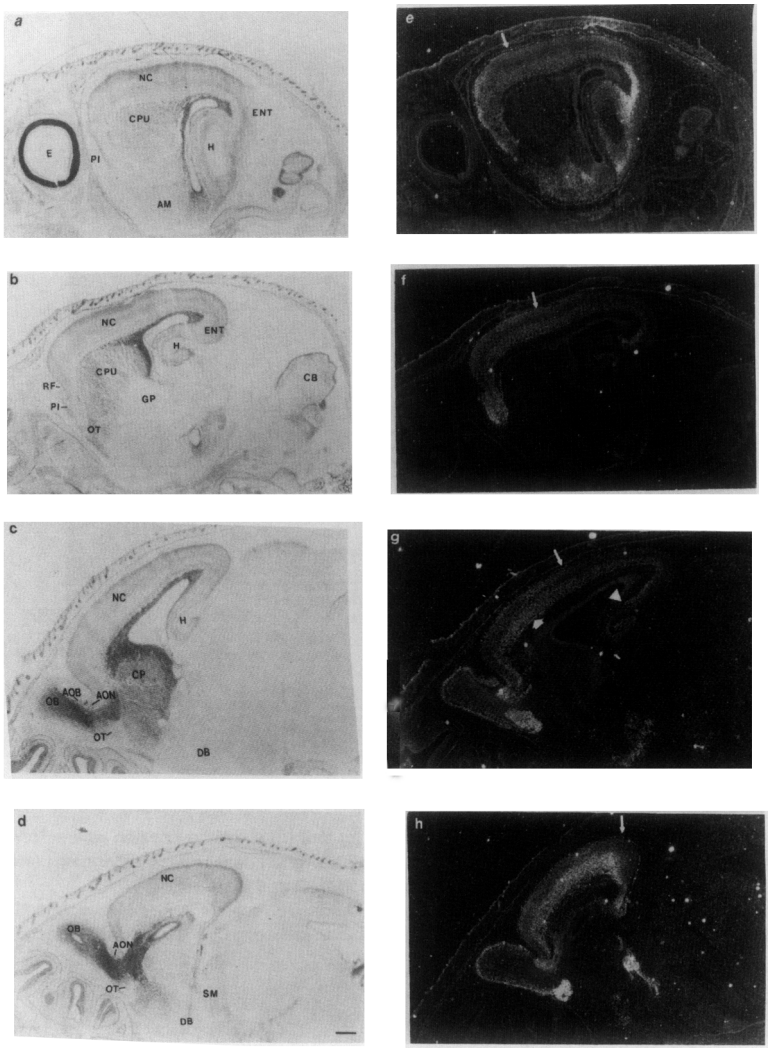


Figure 6. Discontinuity of *Tbr-1* Expression in the Superficial Part of the Cortical Plate at E18.5 Revealed by In Situ Hybridization

(a–d) Toluidine blue stain; (e–h) *Tbr-1* expression. The thin arrows in (e–h) show the position where *Tbr-1* expression ends in the superficial part of the cortical plate. The wide arrowhead in (g) shows *Tbr-1* expression in a layer that probably corresponds to the subplate. The apex of the triangle in (g) shows the discontinuity of *Tbr-1* expression in the region of the subiculum. Bar, 380 μm.

AM, amygdala; AOB, accessory olfactory bulb; AON, accessory olfactory nucleus; CB, cerebellum; CPU, caudoputamen; DB, diagonal bands; E, eye; ENT, entorhinal cortex; GP, globus pallidus; H, hippocampus; NC, neocortex; OB, olfactory bulb; OT, olfacory tubercle; PI, piriform cortex; RF, rhinal fissure; SM, stria medullaris.

Note that the OT in (c) and (d) is, in fact, the AON.

as a part of layer 5 (Neuman et al., 1993). A string of scattered *Tbr-1*-expressing cells can be seen in this region (Figures 7b and 7c). A novel finding is that *Id-2* expression in layer 5 ends abruptly, clearly demonstrating another intracortical rostrocaudal boundary (Figures 7f–7h, arrows). At approximately this position, the pattern of *Tbr-1* expression also changes in layer 5. The thin line of *Tbr-1*-positive cells becomes much wider (Figures 7b and 7c, arrows). The position of this boundary may be at the transition between the frontal (motor) and parietal (sensory) cortical areas (Zilles and Wree, 1995). Superficial to the presumptive layer 5 region, both *Tbr-1* and *Id-2* have less expression; this stratum may correspond to layer 4. Finally, both *Tbr-1* and *Id-2* have strong expression in the superficial strata that probably include layers 1, 2, and 3.

Tbr-1 and *Id-2* expression in the adult brain shows a similar but less distinct result (Figures 7d and 7h). Again, there is a thin strip *Tbr-1*- and *Id-2*-positive cells between the white matter and layer 6 that may correspond to the subplate. Layer 6 remains positive for both genes. Layer 5 also continues to express *Id-2* in a discrete strip that melds into more diffuse expression rostrally (Figure 7h, arrow). On the other hand *Tbr-1* expression in layer 5 is at a low level. Expression of both genes may be low in layer 4, although it is difficult to identify this stratum precisely in our preparations. Both genes are robustly expressed in the superficial zones corresponding approximately to layers 1, 2, and 3.

Tbr-1 *and* Id-2 *Expression in the Thalamus*

During gestation, *Tbr-1* is not expressed in the dorsal thalamus (see Figure 4, Figure 5, and Figure 6). However, between about P1 and P7, thalamic expression of *Tbr-1* begins (Figures 7b and 7c). Weak thalamic expression continues to be present at P17 (data not shown) and in the adult (data not shown). *Id-2* shows a similar postnatal thalamic expression pattern (Figures 7f–7h).

Tbr-1 *Expression Outside of the Forebrain*

Though *Tbr-1* expression is predominantly located in the telencephalon, there is limited expression in other regions as well. In the hindbrain, nests of expressing cells are found in the region of the locus coerulus (data not shown), cerebellar nuclei (Figures 7a and 7b), and Purkinje cells

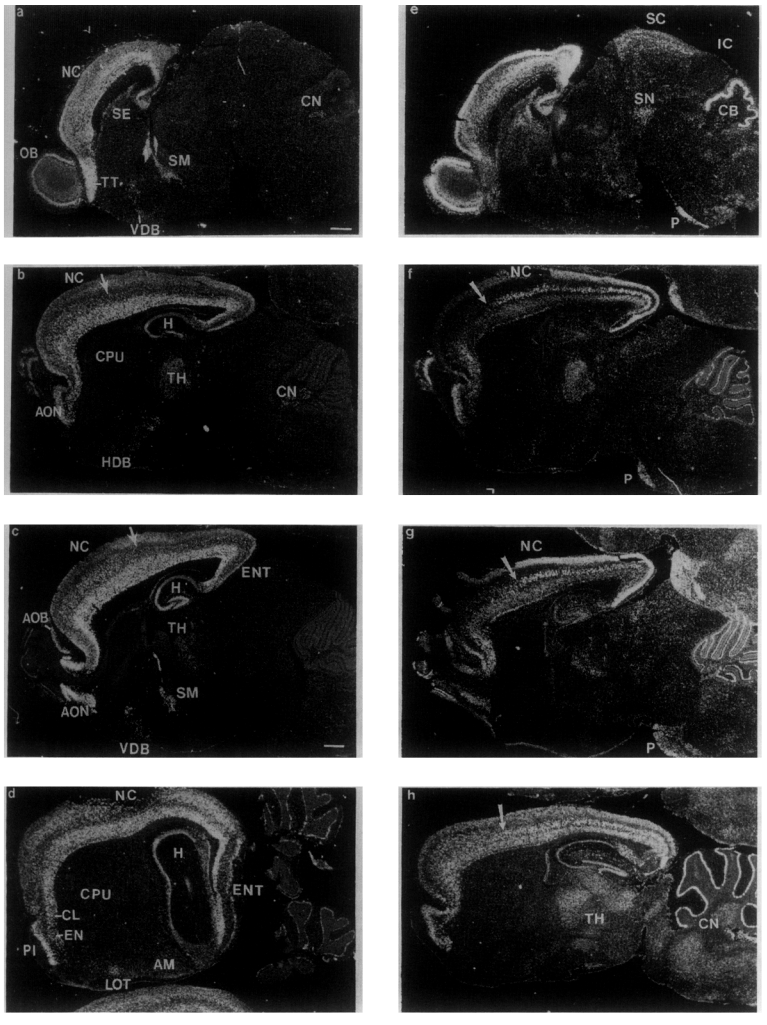


Figure 7. Laminar and Regional Discontinuities of *Tbr-1* and *Id-2* Expression in P1, P7, and Adult Brain Revealed Using Radioactive In Situ RNA Hybridization

(a and e) P1 brain; (b, c, f, and g) P7 brain; (d and h) adult brain.

Tbr-1 expression, left column (a–d); *Id-2* expression, right column (e–h). The arrows in (b), (c), and (f–h) show the position in layer 5 where the band of *Tbr-1* expression becomes wider and *Id-2* expression largely ends. The discontinuities in the superficial cortical layers in (d), (f), and (g) are due to sectioning artifacts.

Bars, 470 μm (a); 600 μm (c).

in the lateral cerebellum (Figure 7d). Non-CNS expression is also apparent in the skin (see Figure 5 and Figure 6) and epithelium of the tongue (data not shown).

Discussion

This paper describes the isolation, nucleotide sequence, and expression of *Tbr-1*, a novel mammalian member of the *Brachyury*/T-box gene family. *Tbr-1* is expressed in a regionally restricted pattern in postmitotic cells of the developing and adult telencephalon. Here we address the proposed function of *Tbr-1*, its relationship to other T-box genes, and how boundaries in *Tbr-1* expression provide evidence for molecularly distinct regions within the embryonic forebrain and, later, within the developing and adult cortex.

The homology of *Tbr-1* to *Brachyury* suggests that it encodes a transcriptional regulator. Because it is not expressed in the VZ, we anticipate that it is not involved in regional specification; rather, we hypothesize that it functions in the control of differentiation.

Tbr-1 Is a Member of the T-Box/*Brachyury* Gene Family

Tbr-1 is the only member of T-box gene family that is extensively expressed in the vertebrate CNS. The high degree of amino acid identity between the mouse and human *Tbr-1* genes (675/682 amino acids) implies that there is a strong selection against altering any of the *Tbr-1* sequence. The other mammalian T-box family members are *Brachyury*, *Tbx-1*, *Tbx-2*, and *Tbx-3* (Herrmann et al., 1990; Kispert and Herrmann, 1993; Bollag et al., 1994). *Brachyury* is expressed in the primitive streak, head process, and notochord; loss of function mutations in this gene lead to caudal mesodermal defects in heterozygotes; homozygotes die during embryogenesis with disruption of their axial and paraxial mesoderm and their neural tube (Herrmann et al., 1990; Schulte-Merker et al., 1992; Kispert and Herrmann, 1993). *Tbx-1* is expressed in the lateral plate mesoderm, branchial arches, otic vesicle, and optic cup; *Tbx-2* is expressed in these regions and the somites and limb bud, whereas *Tbx-3* is expressed in the cells surrounding the foregut, otic vesicle, optic cup, and the nasal pit (Bollag

et al., 1994). Thus, *Tbx-1*, *Tbx-2*, and *Tbx-3* are expressed in a limited region of the forebrain—the optic cups; we have not found evidence for *Tbr-1* expression in the optic cup nor in any of the other regions where the *Tbx* genes are expressed.

Tbr-1 appears to define a new subfamily of T-box-containing genes. Based upon a phylogenetic analysis, we have catagorized four groups of T-box genes: first, *Tbx-1*, *Tbx-2*, *Tbx-3*, and *omb*; second, *Brachyury*; third, *Tbr-1*; fourth, T07C4.2 and T07C4.6 (see Figure 2). *Tbr-1* is closely related to the C. elegans T07C4.2 and T07C4.6 genes, which were incidentally discovered by randomly sequencing the C. elegans genome (Wilson et al., 1994). The expression and function of these genes are not known. Of note, the Drosophila melanogaster *omb* gene is most related to the *Tbx* genes. *omb* is expressed in several structures, including the brain (optic lobes), and loss of *omb* function results in reduction of optic lobe neuropil leading to abnormal optomotor behavior. Thus, *omb* and the *Tbx* genes are expressed in CNS strucutres related to vision.

Tbr-1 Is Expressed in the Preplate and in an A-P Gradient in the Subplate

Tbr-1 is expressed only in postmitotic cells. In the cerebral cortex, expression is first detected around E10.0, demonstrating the time when the first cells leave the cell cycle and begin to differentiate in the preplate (see Figure 3a). There is evidence that the preplate gives rise to the subplate (layer 7) and the marginal zone (layer 1) of the NC, where *Tbr-1* continues to be expressed in the adult (Figure 7d). The subplate is implicated in directing the pattern of innervation of thalamocortical afferents (Allendoerfer and Shatz, 1994).

Tbr-1 is expressed in a rostro-caudal gradient in the subplate at E16.5 and E18.5 (see Figure 5c and Figure 6g), times when the thalamocortical afferents begin to arrive. This gradient probably is due to the known maturational gradient of the cortex (Bayer and Altman, 1991). Bayer and Altman have proposed that the asymmetry generated by maturational (neurogenetic) gradients in the thalamus and cortex provide some of the information used to establish thamalocortical connectivity (Bayer and Altman, 1991). Thus, the gradient of *Tbr-1* expression reflects a molecular difference in the subplate that could participate in regulating thalamocortical patterning.

Tbr-1 and *Dlx-2* Expression at E12.5 Defines Complementary Compartments of Gene Expression: Implications for the Prosomeric Model and Organization of the Dorsal Telencephalon

The prosomeric model hypothesizes that the embryonic forebrain is a segmented structure (Bulfone et al., 1993a; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). The model explicitly defines the longitudinal axis of the forebrain, and recent results with molecular markers support our earlier conclusions (Rubenstein et al., 1994; Shimamura et al., unpublished data). The model also defines boundaries that are transverse and others that are parallel to the axis; these boundaries subdivide the prosencephalon into distinct histogenetic fields. This orthogonal subdivision of the forebrain is consistent with the expression patterns of many genes (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Rubenstein and Puelles, 1994). The model was initially formulated in the absence of molecular markers for the cerebral cortex. Thus, only tentative conclusions were proposed for the subdivisions of the dorsal telencephalon. In this regard, *Tbr-1* has served as a useful gene to begin to define the position of histogenetic boundaries in the cerebral cortex. In this study, we have compared the expression of *Tbr-1* with *Dlx-2* at E12.5. *Tbr-1* is expressed primarily in the cortex (pallium), whereas *Dlx-2* expression is entirely subpallial. The results are summarized and interpreted in three ways (Figure 8).

The schemas show *Tbr-1* and *Dlx-2* expression from a ventrorostral (Figures 8A and 8B) and medial (Figures 8C–8E) perspective. Expression of *Tbr-1* is shown in blue, *Dlx-2* expression in green, and the region where there is overlap of *Dlx-2* and *Tbr-1* expression (diagonal band, VDB and HDB) in pink. The prosomeric model hypothesizes that each histogenetic domain (a neuroepithelial region bounded by two transverse and two longitudinal boundaries that gives rise to a major brain primordia) will be specified by a unique complement of regulatory genes. In this regard, *Tbr-1* and *Dlx-2* expression define different regions of the telencephalon, in that their expression patterns are largely complementary. They share multiple boundaries, such as the pallial/subpallial (NC/LGE) boundary in the telencephalon, and the stria medullaris/supraoptic paraventricular area boundary in the hypothalamus.

Tbr-1 expression in the cortex at E12.5 and E14.5 is continuous, from the primordia of the olfactory bulb through the paleocortex, NC, and archicortex. In our initial formulation of the prosomeric model, we separated various parts of the cortex into distinct segments. However, based on a more detailed topological analysis of the paleocortex and archicortex, as well as considerations of axonal connectivity, we feel that at least two other solutions to the organization of the dorsal telencephalon merit discussion.

Figure 8 shows these three options for the subdivisions of the E12.5 telencephalon; each is compatible with the prosomeric model. Model 1 (Figures 8A and 8C) is the original formulation, which segregates the olfactory bulb, NC, and archicortex into separate segments (p6, p5, and p4, respectively) (Bulfone et al., 1993a; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Here, the secondary olfactory cortical domains (paleocortex) extend into p5 and p4. Likewise, minor parts of the archicortex, such as the indusium grisium and tenia tecta, extend into p5 and p6, respectively (Figure 8C).

The other two variations of the model explore the possibility of incorporating all of the corteces into one prosomere. This interpretation is supported by the relative homogeneity of the *Tbr-1* expression in the E12.5 cortex that spans the archicortical, neocortical, and paleocortical primordia (see Figure 4). In addition, many other genes also show relatively homogeneous expression patterns in the

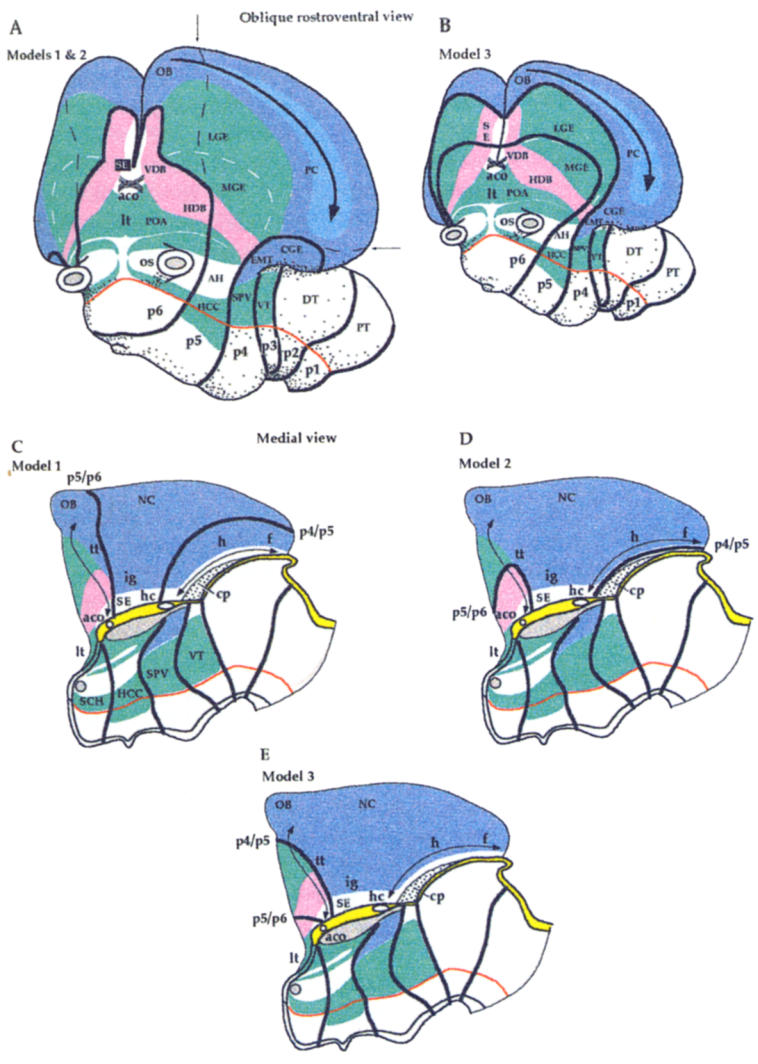


Figure 8. Models of Telencephalic Organization

Schemas showing *Tbr-1* (blue) and *Dlx-2* (green) expression in the E12.5 brain (coexpression of *Tbr-1* and *Dlx-2* is shown in pink) from oblique rostroventral (A and B) and medial (C, D, and E) views. Hypothetical transverse subdivisions of the brain are indicated by black lines. Three alternative models are presented. Model 1 (A and C) segregates the olfactory bulb (OB), NC, and archicortex (including the hippocampus [h]) into separate prosomeres, p6, p5, and p4, respectively. These boundaries are shown as dotted lines in (A). In model 2 (A and D), the entire cortex is in one prosomere (p5), and the pallial/subpallial boundary (cortex/LGE) is a longitudinal limit is p5. In model 3 (B and E), the entire cortex is in one prosomere (p4), and the pallial/subpallial boundary (cortex/LGE) is a transverse limit (p4/p5).

Details in (A) and (B) are as follows: the arrow in the paleocortex shows the trajectory of the olfactory tract; the light blue cortical domain represents the differential expression of Tbr-1 in the lateral telencephalon; the dotted white line shows the boundary between the LGE and MGE. Details in (C), (D), and (E) are as follows: the arrow-tipped line connecting the OB and anterior commissure (aco) corresponds to part of the tract of the anterior commissure; the arrow-tipped line connecting the fimbria (f) and hippocampal commissure (hc) are the hippocampal fibers that give rise to the fornix; the yellow line is the roof plate.

AH, anterior hypothalamus; CGE, caudal ganglionic eminence; cp, chorioid plexus; DT, dorsal thalamus; EMT, eminentia thalami; HDB, horizontal limb of the diagonal band; ig, indusium griseum; LGE, lateral ganglionic eminence; lt, lamina terminus; MGE, medial ganglionic eminence; OS, optic stalk; PC, paleocortex; POA, anterior preoptic area; PT, pretectum; SCH, suprachiasmatic area; SE, septum; SPV, supraoptic/paraventricular area; tt, tectum; VDB, vertical limb of the diagonal band; VT, ventral thalamus.

cerebral cortex at E12.5, including *Id-2* (Zhu et al., 1995), *Wnt-7b* (Bulfone and Rubenstein, unpublished data), *Otx-1* (Simeone et al., 1993; Shimamura and Rubenstein, unpublished data), *Emx-1* and *Emx-2* (Simeone et al., 1992b; Shimamura and Rubenstein, unpublished data), *SCIP* (Frantz et al., 1994a), and *Pax-6* (Stoykova and Gruss, 1994; Shimamura and Rubenstein, unpublished data). Note that most or possibly all of these genes have gradients of expression in the cortex; gradients in gene expression are consistent with the cortex lying within a single developmental field. These gradients may reflect and/or cause neurogenetic (Bayer and Altman, 1991) or morphogenetic processes.

Models 2 and 3 differ in the segment where the cortex is located (in model 2 the cortex is in p5, and in model 3 the cortex is in p4). Model 2 places the entire cerebral cortex in p5 (Figures 8A and 8D), where it is a longitudinal domain dorsal to the LGE. This model also implies that the p4 portion of the telencephalon would consist of the amygdaloid complex ventrally and chorioidal tissues in the roof region, whereas a corresponding region of p6 would consist of the preoptic, diagonal band, and septal territories.

Model 3 places the entire cortex in p4 (Figures 8B and 8E) and largely agrees with the model of Bergquist and Kallen (1954) that assigns separate neuromeric origins to subpallial and pallial parts of the telencephalon. Supporting this model is the observation that many genes recognize the pallial/subpallial (cortex/LGE) boundary, including *Tbr-1* and *Dlx-2* (this paper) as well as *Id-2* (Zhu et al., in press) and *Otx-1* and *Emx-2* (Simeone et al., 1992a, 1992b; Simeone et al., 1993; Shimamura and Rubenstein, unpublished data). In addition, the observation that cortical neuroepithelial cells do not cross this boundary (Fishell et al., 1993) is consistent with the hypothesis that it is an interneuromeric limit. Limits to clonal expansion are associated with interneuromeric boundaries in the hindbrain (Fraser et al., 1990; Birgbauer and Fraser, 1994)

and diencephalon (Figdor and Stern, 1993). However, this experimental data does not unequivocally establish whether the pallial/subpallial boundary is transverse (neuromeric) or longitudinal.

The three models are presented to facilitate the development of experiments that will help elucidate the organization of the telencephalon. Such studies will include further definition of the numbers and boundaries of neuroepithelial domains using gene mapping studies and clonal expansion experiments. These studies should focus on early stages of telencephalic development when its topographical characteristics correlate with the topology of the neural tube. In addition, other approaches are likely to be necessary. For instance, fate mapping studies can define the spatial relationships of regions within the neural plate and neural tube that give rise to specific histogenetic fields (e.g., cortex vs. LGE) and important landmarks (e.g., commissures). Finally, solutions to this problem must consider the topology of the axon tracts that connect the histogenetic territories, as models which create discontinuities or complex topologies for the axonal pathways should be discarded.

Discontinuities in *Tbr-1* and *Id-2* Expression: Are They Molecular Markers for Areal Specification?

Areal subdivisions of the adult cortex can be distinguished on the basis of histology, connectivity, and functional properties. However, only a few molecular markers for these subdivisions have been identified. These include LAMP, a cell surface protein that is expressed at higher levels in allocortical than in neocortical areas (Horton and Levitt, 1988); latexin, a protein that is expressed in the infragranule layers of the lateral cortex (Hatanaka et al., 1994); and a *lacZ* transgene that is expressed in the somatosensory cortex (Cohen-Tannoudji et al., 1994).

In our study of *Tbr-1*, we found regional discontinuities and graded changes in expression of this gene in the cortex. In addition, analysis of *Id-2* (encoding a helix-loop-helix protein) expression also revealed discontinuities in the cortex.

At E12.5, *Tbr-1* shows two stripes of increased expression on the ventrolateral cortical wall (see Figures 3b and 3d). This pattern is located in the cortical region that is likely to become the paleocortex that receives input from the olfactory tract (LaMantia et al., 1993). Although additional studies are needed to investigate the meaning of this striped pattern, it is worth considering the possibility that this pattern relates to cortical subdivisions of the ventrolateral cortex. This region gives rise to paleo and lateral limbic cortical domains; these domains are topologically related to each other as parallel bands. The most ventral stripe includes the piriform and periamygdaloid cortex; dorsal to these are the lateral orbital and ventral agranular insular cortex, and the most dorsal stripe consists of the dorsal and posterior parts of the agranular insular cortex (Zilles and Wree, 1995). Perhaps the most ventral stripe of *Tbr-1* expression corresponds to the paleocortex, and the most dorsal stripe corresponds dorsal and posterior parts of the agranular insular cortex.

Tbr-1 expression also detects regional differences along the rostro-caudal axis of the neocortex beginning around E16.5 and is seen later in gestation. At E16.5 and E18.5, *Tbr-1* expression stops abruptly in the superficial strata of the neocortical plate (see Figure 5 and Figure 6). We do not know whether this discontinuity occurs at the boundary between the primorida of functional zones; however, it is in a position consistent with the boundary between motor and sensory cortex (Zilles and Wree, 1995). These discontinuities are present at a time when there are very few if any thalamic afferents in the superficial strata of the cortical plate (Bicknese et al., 1994). Bicknese et al. (1994) found that the first thalamic afferents enter deep parts of the most mature areas (ventrolateral cortex) of the cortical plate at ~E16.5. This suggests that some aspects of neocortical patterning are intrinsic to this structure.

Postnatally, discontinuities in expression are also apparent, both for *Tbr-1* and *Id-2*. At P7, both *Tbr-1* and *Id-2* show abrupt changes in their expression in neocortical layer 5. *Id-2* has previously been shown to be expressed at high levels in layer 5 of the NC (Neuman et al., 1993). In this paper, we demonstrate that *Id-2* expression in layer 5 ends sharply; this limit may be in the region of the boundary between the frontal (motor) and parietal (sensory cortex) (Figures 7f–7h; Zilles and Wree, 1995). *Tbr-1* expression also changes in this region from a thin to a thick band of layer 5 cells (Figures 7b and 7c). At P7, thalamic inputs are in place; thus, we can not evaluate whether these patterns of expression are influenced by the thalamic afferents. The expression of *Otx-1* also shows a similar discontinuity in its postnatal expression in layer 5 (Frantz et al., 1994b).

Cortex Regional Specification: Protomap or Protocortex?

How are different types of cortex formed? Two models of cortical specification have dominated experimentation in this field for the past decade: the protomap and protocortex models. In its extreme form, the protomap theory proposes that areal specification is controlled by genetically encoded morphogenetic information in the VZ (Rakic, 1988). Thus, groups of neuroepithelial cells in the VZ are exposed to patterning signals that largely predetermine their fate and lead to region-specific histologies. This model depends upon the radial movement of postmitotic cells from the VZ into the cortical plate. In its extreme form, the protocortex model proposes that region-specific histology, connectivity, and functional properties of the cortex are patterned by its thalamic inputs (O'Leary, 1989; O'Leary et al., 1994). This hypothesis is based on the fact that many transplantation and axon rerouting studies have demonstrated the ability of cortical tissue to develop according to different programs depending upon its thalamic input. In addition, retroviral lineage (Walsh and Cepko, 1992, 1993) and other cell migration studies (O'Rourke et al., 1992; Fishell et al., 1993) indicate that at mid-to-late gestation (>E15 in the rat), cells in the VZ, as well as their progeny, are quite mobile, leading to nonradial dispersion. Alternatively, these same studies show a significant per-

centage of the cells do undergo radial migration, and other studies provide additional evidence for radial migration. In addition, other methods have indicated that most cortical neurons obey radial constraints (Nakatsuji et al., 1991; Tan and Breen, 1993). Recently, it has been demonstrated that at the time that the subplate is being generated, there is no detectable tangential migration, thus providing a direct mechanism to transmit morphogenetic information from the VZ to the subplate (D. D. M. O'Leary, personal communication).

There is growing consensus that areal patterning of the cortex involves both prespecification of the neuroepithelium and histogenetic influences from afferent axons. Of course, spatial information must be present in the subplate, and perhaps also the cortical plate, at the time thalamic fibers arrive to determine their proper innervation pattern. Thus, one of our goals has been to determine whether there is molecular evidence for regionalization of the cortex prior to the arrival of thalamic afferents. The answer is mixed. At E12.5, there are clear differences in *Tbr-1* expression in the neocortical and the ventrolateral areas that give rise to the paleocortex and limbic cortex (see Figures 3b and 3d). However, this result may not have relevance to neocortical specification. In addition, at E12.5 and E14.5, *Tbr-1* expression in the NC is relatively homogeneous, except for an A–P gradient in the thickness of the cortical plate (see Figure 4e). However, on E16.5 and E18.5, *Tbr-1* expression ends sharply in the upper layers of the cortical plate (see Figure 5 and Figure 6). As noted earlier, thalamocortical fibers only begin arriving to the cortical plate at E16.5 (Bicknese et al., 1994). This is evidence for molecular heterogeneity in different areas of the NC, supporting the hypothesis that some aspects of regionalization are regulated in the absence of thalamic input.

Tbr-1 and *Id-2* Are Preferentially Expressed in Particular Cortical Laminae

Patterning of the CNS is a three-dimensional problem. One can conceptualize regional specification as a process requiring two-dimensional spatial information in the plane of the neuroepithelium (Rubenstein et al., 1994). The third dimension of structural complexity is generated as cells leave the mitotic cycle and migrate towards the pial surface; cells of different fates arrest their migrations in different positions. In the cortex, differential migration leads to a laminar histology. There is now an active search for the cellular and molecular mechanisms that generate this laminar structure. One approach has been to identify genes that are candidates for regulating this process. For instance, a candidate gene would encode a regulatory molecule, such as a transcription factor, that is expressed in only one cortical layer. We are not aware of any molecule of this type, although there are several transcription factors that are preferentially expressed in specific cortical layers. These include *Otx-1* (layers 5 and 6; Frantz et al., 1994b), *SCIP* (layers 2, 3, and 5; Frantz et al., 1994b), *Id-2* (all layers except layer 4 based upon results from this paper; layers 2, 3, and 5 based on Neuman et al., 1993), and *Dlx-2* (marginal and subventricular zones late in development; Porteus et al., 1994). In this paper, we showed that while *Tbr-1* is probably expressed in all cortical layers its expression is much more pronounced in the deep (subplate, layer 6, and probably part of layer 5) and superficial laminae (probably layers 3, 2, and 1; Figures 7b–7d). In addition, we confirmed and extended the results with *Id-2*, showing that this candidate transcriptional regulator is also preferentially expressed in specific layers (see Results and Figures 7f–7h for details). Thus, because these genes are expressed in multiple layers, it is unlikely that they regulate laminar fate. However, the results are consistent with the hypothesis that *Tbr-1* and *Id-2* are involved in differentiation of particular cell types and the maintenance of their phenotypes in the adult brain.

Tbr-1 Expression in the Thalamus Begins Postnatally

The pattern of *Tbr-1* expression is remarkably stable from embryogenesis into adulthood, except for one area—the thalamus. Prior to P7, we have not detected *Tbr-1* expression in the thalamus. On P7 (Figures 7b and 7c) and P16 (data not shown), *Tbr-1* thalamic expression is readily detectable. This incidental finding is intriguing because of the close functional relationship of the cortex and the thalamus. *Id-2* shows a similar late onset of thalamic expression (Figures 7f–7h). Perhaps *Tbr-1*-expressing cells produce a secreted substance that can induce its own expression. Thus, by P7, when the thalamocortical circuit has largely been established, a signal from the cortex is transmitted through cortical axons from the deep layers (where *Tbr-1* and *Id-2* are strongly expressed) and is secreted at thalamic synapses. Expressing the same transcription factor in both the presynaptic and postsynaptic cell could coordinate expression of proteins that stablize cell–cell interactions, such as homophilic adhesion molecules.

Speculations on the Genetic Hierarchy that Patterns Cortex Development

The morphogenetic signals and the responding transcription factors that pattern the cortex are unknown, although candidate molecules such as *Tbr-1* are rapidly being discovered. Because *Tbr-1* is expressed in postmitotic cells, it must be downstream of transcription factors that are expressed in the proliferative zone. Thus, one can begin to make hypotheses about the hierarchy of regulatory genes involved in cortical regional specification and differentiation. There are a number of transcription factors that are expressed in the VZ. These include homeobox genes (*Otx-1*, *Emx-1*, *Emx-2* [Simeone et al., 1992a, 1992b, 1993], and *Pax-6* [Walter and Gruss, 1991; Stoykova and Gruss, 1994]), as well as nonhomeobox transcription factors (*BF-1* [Tao and Lai, 1992] and *N-Myc* and *Heir-1* [Ellmeier et al., 1992]). These genes are candidate regulators of *Tbr-1*. In the future, genetic manipulations will begin to decipher the regulatory network that controls *Tbr-1* expression. Finally, we hypothesize that *Tbr-1* encodes a transcription factor because of its homology to *Brachyury*. Thus, future studies will be directed towards identifying

genes that are regulated by *Tbr-1*. In this vein, we are mutating *Tbr-1* to determine its role in cortical development and eventually to study the molecular basis for its function.

Experimental Procedures

Cloning and Sequence Analysis of the Murine and Human *Tbr-1* cDNA

Tbr-1 was initially identified from a subtracted cDNA library that is enriched for genes that are transcribed at higher levels in the E14.5 mouse telencephalon than in the adult telencephalon (Porteus et al., 1992). Random clones from this library have been studied by in situ RNA hybridization to E14.5 embryo sections. *Tbr-1* was noted because of its strong expression in the telencephalon. The initial clone (*Tes-56*) contained a 264 bp insert. Longer clones were isolated by screening cDNA libraries prepared from E14.5 telencephalon (Porteus et al., 1992). Fifty independent isolates were obtained; the longest clone was 3.805 kb.

The human *Tbr-1* ortholog was isolated by screening a 17 week fetal brain cDNA library (Stratagene) using the mouse *Tbr-1* as a probe. Two independent isolates were obtained (3.0 and 1.2 kb).

Both strands of the coding sequence of the human and mouse *Tbr-1* cDNA clones were sequenced using the dideoxy method using the Sequenase kit (USB). Oligonucleotide primers were generated every ~200 bp to allow us to obtain overlapping sequence information on each strand. Nucleotide sequence analysis was performed using the Eugene and SAM programs (MBIR, Baylor College of Medicine). Dendogram analysis was performed using the Pileup program (GCG).

Analysis of *Tbr-1* Expression: Northern and In Situ Hybridization

Northern analysis was performed as described in Sambrook et al. (1989). Total RNA (10 μg) from E14.5 and adult telencephalons was electrophoresed and transferred to Duralon membranes (Stratagene). The *Tbr-1* transcript was detected using a random-primed radioactive probe made from the extreme 3′ end of the *Tbr-1* clone (a 264 bp fragment; entirely untranslated sequence).

Tbr-1 expression was detected in tissue sections from whole mouse embryos and fetuses, as well as in postnatal brains using radioactive in situ RNA hybridization (Bulfone et al., 1993a). The antisense *Tbr-1* riboprobe was transcribed from a *Tbr-1* cDNA clone encoding the most 3′ 264 bp. *Id-2* expression was detected using a riboprobe from the ~440 bp at the 3′ end of a cDNA clone (M. Israel, UCSF). *Dlx-2* expression was detected as described in Bulfone et al. (1993a). Quantitation of the level of hybridization in specific regions of the NC was performed by counting the number of silver grains within an area specified by an eye piece reticule (16 μm^2), using a 40× objective on a Nikon Optiphot microscope. At least 30 16 μm^2 areas were counted for each region studied. The mean number and the SD of grains per box was determined. The numbers were corrected by subtracting the background level of hybridization found in the striatum (4 ± 2 grains per 16 μm^2).

In situ hybridization to whole embryonic brains (E10.0–12.5) was performed using nonradioactive methods according to the methods of Conlon and Rossant (1992) and Shimamura et al. (1994). In brief, embryos were collected in ice cold PBS and fixed with 4% paraformaldehyde/PBS overnight, dehydrated in an ascending series of methanol solutions, and stored in methanol at −80°C. Embryos older than E10.0 were subsequently bleached by incubation in a mixture of methanol and hydrogen peroxide (5:1), followed by rehydration through a descending series of methanol. The samples were then transferred to 0.1% of Tween-20 (PBT). The brains were dissected from the embryos at this stage. These samples were treated with 10 mg/ml of proteinase K for 10 min at room temperature. Then, the embryos were hybridized with 1 mg/ml of digoxigenin-labeled and/or fluorescein-labeled riboprobes for 12 hr at 63°C, followed by washing and treatment with RNases. The hybridized probes were detected by anti-digoxigenin and anti-fluorescein antibodies using an alkaline phosphatase reaction.

BrdU Labeling and Detection

A solution containing BrdU was injected into the peritoneal cavity of pregnant mice 1 hr before sacrificing by cervical dislocation (Porteus et al., 1994). The embryos were prepared and sectioned in a manner identical for in situ RNA hybridization (Bulfone et al., 1993a). Nuclei containing BrdU-labeled DNA were detected using an anti-BrdU antiserum. Nuclei that bound these antibodies were detected using the peroxidase method (Vector ABC kit).

Chromosomal Localization of *Tbr-1*

Tbr-1 was mapped on the mouse genome by performing a linkage analysis using 50 DNA samples from the European Collaborative Interspecific Backcross (European Backcross Collaborative Group, 1994). This mapping panel was derived from DNA isolated from a Mus musculus (C57Bl/6) and Mus spretus intercross and backcross. Data from the mapping panel can be accessed through the World Wide Web (http//www.hgmp.mrc.ac.uk/local-data/mbx/Mbx__homepage.ht ml). *Tbr-1* has the designation 94/PE/001 in this database.

To localize *Tbr-1* using this mapping panel, we identified a polymorphism between Mus spretus and Mus musculus (C57Bl/6) in the 3′ untranslated region of the *Tbr-1* gene using single-strand chain polymorphism analysis (Orita et al., 1989). An end-labeled, 250 bp fragment was generated using PCR and two oligonucleotide primers designed from the *Tbr-1* sequence (5′ to 3′, ACCACTGTGTGCCCTGGT; 5′ to 3′, TAAAGGTGGAGTGGGGTCTG). This polymoprphism was used to determine the alleles (spretus or musculus) inherited by 50 mice from the European Backcross Collaborative Group panel. Comparison of this data to the inheritance of simple sequence length polymorphism loci previously determined for these mice (Dietrich et al., 1994) was used to detect linkage for *Tbr-1* on chromosome 2. LOD scores >>3 were obtained from pairwise linkage analysis for both D2MIT6 and D2MIT11, with a peak LOD score at 32 cM on the European Backcross Collaborative Group map.

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