Regulatory Gene Expression Boundaries
Demarcate Sites of Neuronal Differentiation
in the Embryonic Zebrafish Forebrain

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Summary

During development of the zebrafish forebrain, a simple scaffold of axon pathways is pioneered by a small number of neurons. We show that boundaries of expression domains of members of the eph, forkhead, pax, and wnt gene families correlate with the positions at which these neurons differentiate and extend axons. Analysis of genetically or experimentally altered forebrains indicates that if a boundary is maintained, there is appropriate neural differentiation with respect to the boundary. Conversely, in the absence of a boundary, there is concomitant disruption of neural patterning. We also show that a strip of cells within the dorsal diencephalon shares features with ventral midline cells. This strip of cells fails to develop in mutant fish in which specification of the ventral CNS is disrupted, suggesting that its development may be regulated by the same inductive pathways that pattern the ventral midline.

Introduction

One of the earliest steps in the formation of a functional nervous system is the acquisition of regional identity by cells in different areas of the CNS. In recent years, many of the genes that may be involved in regional patterning of the CNS have been identified. For instance, it seems likely that genes of the Hox family are involved in the acquisition of regional identity by cells in the developing hindbrain (Krumlauf et al., 1993). This region of the CNS develops as a series of segmental units termed rhombomeres, and different combinations of Hox gene expression may regulate the individual identities of each rhombomere.

It remains unclear how studies of hindbrain development relate to more rostral brain regions, and although the last few years have seen a considerable increase in the number of genes that may be involved in forebrain morphogenesis, there is still no consen-
Figure 1. *pax6*, *rtk1*, *rtk2*, *axial*, and *wnt1* Expression in the Developing Forebrain
Dorsal views of whole-mounted brains hybridized with digoxigenin-labeled RNA probes (except *Dj* or antibody (D); rostral is up. (A) *pax6*, 8 som; (B) *rtk1*, 20 som; (C) *rtk2*, 14 som; the arrowhead indicates a thin strip of *rtk2* expression in the midbrain; (D) *pax6*, 18–20 som; in the hindbrain, expression is prominent at rhombomere boundaries (arrowheads); (E) *pax6*, 30 som; (F) *rtk1*, 30 som; (G) *rtk2*, 30 som; one eye was removed during preparation; (H) *axial* plus *wnt1*, 30 som; the midbrain/hindbrain boundary stripe of *wnt1* expression (black arrowhead) and anterior strip of *axial*-expressing cells (white arrowhead) are indicated. fb, forebrain; hb, hindbrain; l, lens; mb, midbrain; nr and tr, nasal and temporal retina; op, olfactory placode; os, optic stalk; r1–r3, rhombomeres 1–3; t, telencephalon; tv, III and IV, tectal, third and fourth ventricles. Bar, 100 μm.

Figure 2. *pax6*, *rtk1*, *rtk2*, *axial*, and *wnt1* Expression in the Developing Forebrain
Lateral views of whole-mounted embryos labeled with RNA probes (B–H) or antibody (A). Rostral is to the left and dorsal is up. In each panel, a ventral dot indicates the position of the incipient ventral flexure and a dorsal dot, the anterior boundary of the epiphysis. Arrowheads in (A), (B), (E), and (F) indicate gaps in expression. (A) *pax6*, 13
as a proto-oncogene overexpressed in a variety of carcinomas (Hirai et al., 1987). The first indication that receptors of this family may be involved in spatial patterning of the CNS came from the observation that the expression domains of the mouse gene Sek included rhombomeres 3 and 5 (Nieto et al., 1992). In this study, we examine two eph-related RTKs; rtk1 is the zebrafish homolog of Sek, and rtk2 is a novel member of the eph family (Xu et al., 1994).

By combining in situ hybridization with immunocytochemistry, we show that the earliest neurons in the forebrain differentiate along boundaries of gene expression. The pathways that these neurons pioneer also develop at boundaries, in some cases developing precisely along the boundary, in others, separated from it by one or two cells. Analysis of spatial relationships among boundaries, neurons, and axons in genetically or experimentally altered brains supports the notion that there is a causative link between gene expression boundaries and sites of neuronal differentiation and axogenesis.

Results

Spatially Restricted Gene Expression Domains in the Developing Forebrain

During development of the zebrafish forebrain, the expression patterns project axons between 16–18 hr postfertilization (14–18 somites [som]), and by 24 hr (30 som), the major subdivisions of the brain can be recognized. All sites of neurogenesis, neuronal differentiation, and axogenesis within the forebrain have been described (Chitnis and Kuwada, 1990; Wilson et al., 1990; Ross et al., 1992).

We have defined the expression domains of six genes, axial, pax6, pax2, rtk1, rtk2, and wnt1, with respect to sites of neuronal differentiation and axogenesis in the forebrain. Previous descriptions of pax5, pax2, and wnt7 expression (Krauss et al., 1991a, 1991b, 1991c; Molven et al., 1991; Püschel et al., 1992a, 1992b) were insufficient to evaluate the expression domains of these genes with respect to sites of neurogenesis, so the analysis was repeated. Figure 1 and Figure 2 show dorsal and lateral views of gene expression domains during early forebrain morphogenesis.

pax6 is widely expressed in cells contributing to the dorsal forebrain from the end of epiboly. By 8 som, high levels of pax6 expression are detected near the forebrain/midbrain boundary with transcript levels declining toward the anterior tip of the brain (Figure 1A). From about 12 som, transcripts are present throughout the eyes, whereas little or no expression is detectable in the optic stalks (Figure 1D). pax6 expression gradually resolves into several discrete domains. Although the ventral boundary of pax6 expression initially lies approximately parallel to the ventral surface of the brain, a dorsally directed strip of nonexpressing cells gradually becomes apparent in the mid-diencephalon (the midsagittal boundary; Figures 2A and 2B). By 28 som, high levels of transcripts are detected in dorsal thalamus, ventral thalamus ventral to the optic recess/third ventricle, and in a cluster of telencephalic neurons (Figure 1E and Figure 2B). Although initially expressed throughout the roof of the diencephalon, by 20 som, dorsal midline cells no longer express pax6 (Figure 1D).

From the end of epiboly, rtk1 is expressed throughout the anteroposterior extent of the dorsal forebrain, including the proximal portions of the optic stalks but not the eyes (Figures 1B and 1F). The ventral and caudal boundaries of rtk1 expression are similar to those of pax6 (compare Figures 2E and 2A). Indeed the rtk1 expression domain is also divided by a strip of nonexpressing cells at the midsagittal boundary (Figure 2F). By 30 som, the dorso/anterior boundary of rtk1 expression is parallel but several cells dorsal to the comparable pax6 expression boundary (compare Figures 2B and 2F). Low levels of rtk1 transcripts were also detected at and adjacent to the ventral midline.

By 14 som, rtk2 transcripts are present in a band of tissue at, and rostral to, the forebrain/midbrain boundary, overlapping with the caudal-most extent of rtk1 and pax6 expression (Figure 1C and Figure 2G). Transcripts are also detectable in the telencephalon and anterior optic stalk (Figure 2G), in the temporal half of the eye (Figure 1G) and transiently in the mid-diencephalon (Figure 1C and Figure 2C). From 30 som, rtk2 is more widely expressed in the forebrain (Figure 2H).

Within the CNS, axial is initially expressed in ventral midline cells (Strähle et al., 1993). By 12 hr, the anterior boundary of axial expression coincides with the future site of the ventral flexure. From this position, expression spreads dorsally along a finger of cells in the mid-diencephalon. The tip of this finger eventually underlies the anterior epiphysis (Figure 1H; Figures 2C and 2D) and overlies the zone within which pax6 and rtk1 are expressed at low levels. Comparative analysis (see, for example, Figures 2B and 2D) indicates that the axial and pax6 expression domains do not overlap. In older embryos, the strip of axial-expressing cells is displaced rostral to the epiphysis.
Figure 3. Gene Expression Boundaries Demarcate Sites of Neuronal Differentiation and Axogenesis in the Anterior Forebrain
Lateral views of whole-mounted brains with eyes removed and rostral to the left (except [G] and [H]). (A) Anti-AT labeling of axon pathways at 28 som; (B) anti-GABA labeling of the principal neuronal nuclei of a 30 hr embryo; segmental clusters of neurons are apparent in the hindbrain (arrowheads); (C) combined anti-pax6 and anti-AT antibody labeling of a 13 som embryo; the first differentiated neurons in the nMLF are indicated (arrowheads); (D) anterior forebrain of 16/17 som embryos labeled with both an RNA probe to rkt1 (blue) and HNK1 antibody (brown); the first neurons to differentiate in the telencephalon (white arrowheads) are beginning to develop HNK1 immunoreactivity along the dorso/anterior rkt1 expression boundary, and the first neurons in the nTPOC (arrows) are differentiating and sending axons along the ventral rkt1 expression boundary; (E and F) 28 som embryos labeled with RNA probes to rkt1 (E) or pax6 (F) (blue/purple) and HNK1 (brown); (G and H) frontal views of 28 and 30 som embryos labeled with anti-pax2 antibody (brown) and anti-AT antibody (grey, axons). AC and tAC, anterior commissure and its associated tract; cb, cerebellum; dd, dorsal diencephalon; e, epiphysis; hy, hypothalamus; m, midbrain; MLF and nMLF, medial longitudinal fasciculus and its nucleus; or, optic recess; os, optic stalk; POC, postoptic commissure; SOT, supraoptic tract; t, telencephalon; te, tectum; Tn, telencephalic nucleus; tPC, tract of the posterior commissure; TPOC and nTPOC, tract of the postoptic commissure and its nucleus. Bar, 50 μm (A, C, G, and H), 70μm (B), 35 μm (D, E, and F).
Spatially restricted wnt1 transcripts are first detected within the CNS from the end of gastrulation. The gene is expressed within the roof of the midbrain and at the cerebellar fold, confirming previous results (Mollen et al., 1991). The anterior limit of expression is beneath the ependyma above the most dorsal axonal expressing cells (Figure 2C).

**Boundaries of Gene Expression Domains Define Sites of Neuronal Differentiation and Axon Pathway Formation**

The three principal sites of early neuronal differentiation in the rostral brain of the zebrafish embryo are a nucleus of cells in the telencephalon, the nucleus of the tract of the postoptic commissure (nTPOC), and neurons in and adjacent to the nucleus of the median longitudinal fasciculus (nMLF; Figure 3B; see Wilson et al., 1990; Ross et al., 1992). A small number of neurons also differentiate in the ependyma and nucleus of the posterior commissure. As all axon pathways in the rostral brain are pioneered by neurons that lie along their length, the distribution of axons reflects the distribution of underlying neurons (Figure 3A).

It has been proposed that axon pathways may be located at boundary zones in the CNS (Krauss et al., 1991b; Fidgter and Stern, 1993; Wilson et al., 1993). To test this hypothesis, we examined the spatial relationships among sites of neuronal differentiation, axonogenesis, and regulatory gene expression boundaries. This was done by examining embryos (n > 100) in which both neurons and regulatory gene transcripts or protein had been visualized simultaneously.

Boundaries of pax6 expression at 13 som approximate the positions at which axon pathways are pioneered within the following hours (compare Figures 3C and 3A). For instance, at 15 hr, the dorso/anterior limit of pax6 expression within the telencephalon borders directly the region that is beginning to undergo neuronal differentiation. However, this relationship is transient in that by 24 hr, the dorso/anterior boundary of pax6 expression has receded from the telencephalon to a boundary ventral to the optic recess/third ventricle (Figure 3F), and the gene is upregulated in many of the differentiating telencephalic neurons. Thus, temporal alterations in pax6 expression mean that not all of the pax6 expression boundaries continue to delineate axon pathways after the pathways have formed.

Unlike pax6, the dorso/anterior boundary of rtk1 expression abuts directly the band of differentiating telencephalic neurons and defines the pathway taken by axons that pioneer the anterior commissure throughout the early period of neuronal differentiation (Figures 3D and 3E). rtk2 is expressed in telencephalic cells adjacent and dorso/anterior to cells that express rtk1, including the band of differentiating telencephalic neurons (data not shown).

The first neurons to differentiate in the TPOC lie along the boundary between the optic stalk and the postoptic region of the diencephalon. This zone is the ventral expression boundary of rtk1 (Figure 3D) and is initially also the ventral limit of pax6 expression. However, by 24 hr, pax6 expression only demarcates this boundary caudal to the optic stalk and supraoptic tract (Figure 3F). By the time the TPOC has been pioneered, there is a dorsally directed gap of pax6 and rtk1 expression at the middiencephalic boundary (Figure 3D). This is approximately the location at which TPOC axons deflect to extend toward the nMLF (Figure 3A; see also Wilson and Easter, 1991b).

By 24 hr, a few cells in the nTPOC have extended rostrally directed axons that pioneer the postoptic commissure. The pathway taken by the commissural axons is demarcated by the ventral expression boundary of pax2 at the junction of the optic stalk with the postoptic diencephalon (Figures 3G and 3H).

The nMLF is an elongated cluster of reticulospinal neurons located caudal to the ventral flexure (Figure 3B). It is usually presumed to be a midbrain nucleus based on its position caudal to the ventral flexure, which has been widely described as the ventral limit of the boundary between forebrain and midbrain (the dorsal limit is at or near the posterior commissure). However, both gene expression boundaries and the position of the tract of the posterior commissure (TPC: Figure 3A and see Figure 2) suggest that the ventral limit of the forebrain/midbrain boundary is located caudal to the ventral flexure. Using this definition, the nMLF straddles the ventral boundary between forebrain and midbrain. Its rostral extent overlaps the caudal boundary of pax6 and rtk1 expression (Figures 4A and 4B).

The nMLF is located along the ventral boundary of pax6, rtk1, and rtk2 expression and the dorsal boundary of axial expression (Figure 4; rtk2 not shown). Some of the nMLF neurons express axial, whereas others express pax6, indicating that the nucleus lies at the boundary between the expression domains of these two genes (Figures 4C and 4D). Between 26 and 30 som, neurons in the vicinity of the nMLF send dorsally directed axons that pioneer the tract of the posterior commissure 1–2 cell diameters inside the caudal boundary of the pax6 expression domain (Figures 4E–4G).

There are two other early axon pathways in the forebrain that we do not describe in detail in this study. The supraoptic tract lies along the dorsal edge of the optic stalk (see Figure 3A). The dorsoventral diencephalic tract (Wilson and Easter, 1991a, 1991b) arises from a small number of neurons that differentiate along the dorsal edge of pax6 and wnt1 expression domains (see Figures 7C and 7D). This tract runs ventrally from the ependyma parallel to the rostral boundary of axial expression and intersects the TPOC dorsal to the ventral flexure.

**Neuronal Differentiation is Perturbed in Embryos Lacking the Ventral pax6 Expression Boundary**

**cylops** is a recessive mutation that affects the specification of ventral midline structures in the CNS (Hatta
et al., 1991). Homozygous mutant embryos lack a floor plate in the spinal cord and hindbrain and exhibit fusion of the eyes in the forebrain.

We examined the forebrain of cyclops mutant embryos for alterations in gene expression boundaries, reasoning that if boundaries influence neural patterning then boundary changes should produce concomitant changes in neurons or their axons. In contrast to wild-type embryos, pax6 is expressed throughout the dorsoventral extent of the diencephalon of cyclopic embryos (Figure 5C; see also Hatta et al., 1994). Prior to 15 hr, the cross-sectional area of the diencephalon is not significantly reduced in the mutant (Figure 5C) as compared with wild type (see Figure 6B), suggesting that pax6 may be ectopically expressed in ventral cells, as opposed to all ventral cells being absent.

As the nTPOC and its tract normally form along the ventral boundary of pax6 expression, we examined what happens to this nucleus in cyclops mutants in which this boundary is disrupted. In support of a possible functional role for boundaries in neural patterning, we were unable to detect any differentiated neurons in the ventral diencephalon (Figures 5D, 5E, and 5G; see also Hatta et al., 1994).

The Cyclops Mutation Affects Cells in Dorsal Parts of the Middiencephalon

As described above, there is a discontinuity in the expression domains of genes along a strip of cells
Figure 5. The cyclops Mutation Perturbs Boundary Formation and Neuronal Differentiation in the Forebrain

Whole-mounted brains (except B and C) with rostral to the left (except C).
(A–C) 12 som whole-mounted (A), sagittal (B), and transverse (C) sections through the diencephalon of cyclops mutant embryos labeled with anti-pax6 antibody. A dorsally directed furrow (arrowhead) is present in the middiencephalic neuroepithelium.
(D) Sagittal hemisection of a 30 som cyclops mutant embryo labeled with anti-pax6 (brown) and anti-AT (black) antibodies. The only immunoreactive neurons in the ventral brain are at the caudal boundary of pax6 expression (arrows).
(E) GABA expression in a 30 hr cyclops mutant embryo. A normal population of GABA-positive cells are present in the telencephalon and rhombomeres (arrowheads), reduced numbers of cells are present in the nMLF (out of focus, arrow), but no nTOC neurons are present.
(F) Sagittal hemisection showing a few axial-expressing cells (arrowhead) at the tip of the furrow of a 25 hr cyclops mutant embryo.
(G) Axon pathways labeled with anti-AT antibody in a 30 som cyclops mutant embryo. The supraoptic tract makes a loop around the ventral diencephalon.
(H) DII labeled epiphysial projection (arrowheads) in 27 hr cyclops mutant embryo.

at the middiencephalic boundary. Genes expressed widely in dorsal tissue are not expressed at this boundary, whereas axial, a gene expressed elsewhere in ventral midline cells is (see Figure 2). The cyclops mutation is thought to prevent specification of ventral nervous tissue, so if the strip of cells at the middiencephalic boundary does constitute a cluster of cells sharing features with ventral midline tissue, it should be disrupted in cyclops mutant embryos.

In 12–14 som cyclops mutants, we observed a dorsally and medially directed furrow in the neuroepithelium at the middiencephalic boundary (Figures 5A and 5B) that by 24 hr had extended to beneath the anterior epiphysis (Figure 5D). As axial is normally expressed within cells at the middiencephalic boundary, we examined the expression of this gene in cyclops mutants. Although neural expression of axial is absent in 8 som cyclops mutant embryos (Strähle et
Figure 6. Lithium Treatment Disrupts Patterning of Dorso/Anterior Forebrain

Whole-mounted embryos treated with LiCl, with rostral to the left (except [B]) and dorsal up.

(A–C) Lateral (A), frontal (B), and dorsal (C) views of 12–14 som lithium-treated embryos labeled with anti-pax6 antibody. In (A), the reduced eyes are out of the plane of focus.

(D) HNK1-labeled ectopic neuron (arrow) in an eyeless embryo.

(E–I) 30 som lithium-treated embryos labeled with both anti-pax6 (brown) and anti-AT (black) antibodies.

AC, anterior commissure; cb, cerebellum; d, diencephalon; dd, dorsal diencephalon; e, epiphysis; fp, floor plate; h, hypothalamus; hb, hindbrain; mb, midbrain; MLF and nMLF, medial longitudinal fasciculus and its nucleus; or, optic recess; os, optic stalk; PC and TPC, posterior commissure and its associated tract; POC and TPOC, postoptic commissure and its associated tract; se, surface ectoderm; t, telencephalon; te, tectum; vf, ventral flexure; vtc, ventral tegmental commissure. Bar, 50 μm (A–G), 25 μm (H–I).
al., 1993), we found that by 24 hr, axial expression was detected in cells scattered along the ventral midline of the spinal cord (data not shown) and in one or two cells at the midline tip of the furrow (Figure 5F; see Krauss et al. (1993) for similar results with shh).

This middiencephalic furrow in cyclops mutant embryos caused two major axon pathway defects. First, upon exiting the supraoptic tract, telencephalic axons were unable to extend toward the nMLF, and although a few axons coursed dorsally up and around the furrow, the majority formed a commissure around the top of the fused eye (Figure 5G; see also Hatta et al., 1994). Second, epiphyseal axons that normally extend ventrally and turn rostrally midway down the diencephalon (Wilson and Easter, 1991b) were prevented from making rostral turn and meandered caudally along the ventral midline (Figure 5H).

**Lithium Treatment Disturbs Development of the Dorso/Anterior Forebrain but Does Not Affect the Coincidence of Axon Pathways with Boundaries**

Previous studies have shown that LiCl administered at the midblastula stage causes brain abnormalities, including a loss of eyes (Yamaguchi and Shinagawa, 1989; Stachel et al., 1993). Since eyes are dorso/anterior structures (Puelles and Rubenstein, 1993), we reasoned that lithium treatment may provide a route to perturb dorso/anterior development of the forebrain, complementary to the ventral defects found in cyclops embryos. Treated embryos (n > 300) showed variations in the severity of defects dependent upon the duration of immersion in LiCl. The least affected embryos showed normal or slightly reduced eye size and occasional ectopic sensory neurons (Figure 6D). As severity increased, eye size reduced further, and defects in dorsal forebrain became apparent. The most severely affected embryos lost all dorso/anterior forebrain structures and showed reduction in ventral structures. By morphological examination of the remaining ventral brain, we confirmed that lithium treatment primarily perturbed dorso/anterior tissue. Floor plate tissue was unaffected (Figure 6H), and others than in severely affected embryos, hypothalamic tissue was present, although sometimes reduced in size (Figure 6I).

The distribution of pax6 protein in lithium-treated embryos was essentially normal in what remained of the forebrain (n > 50; Figures 6A–6C). For instance, expression was restricted to dorsal tissue as in normal embryos (Figure 6B), and when eyes were reduced in size, pax6 expression was nevertheless appropriate to the remaining tissue (Figure 6C).

To analyze neuronal and axonal distribution with respect to pax6 expression, embryos were labeled with antibodies to both pax6 protein and neurons (n > 50). In the mildly affected embryo shown in Figure 6E, forebrain pattering is almost normal, though there is a slight reduction in the size of the optic stalk and a concomitant alteration in the position of the postoptic commissure with respect to the anterior commissure (compare Figure 6E with Figure 3A). Figure 6F shows an embryo that lacks eyes and telencephalon so that the epiphysis connects directly to the hypothalamic region. The TPC occupies a normal position with respect to the caudal expression boundary of pax6. Figure 6G shows a severely affected embryo lacking telencephalon and much of the dorsal diencephalon. However, despite the domain of pax6 expression being greatly reduced, axons in the TPC still trace a course 1–2 cell diameters inside the pax6 expression domain. The most severely affected embryos lacked any forebrain pax6 expression and did not possess a TPC (data not shown). Therefore, despite severe disruptions of dorso/anterior forebrain tissue in lithium-treated embryos, axon pathways and gene expression boundaries maintained appropriate relationships in the remaining CNS tissue.

**Discussion**

Little is currently known about the molecular mechanisms that underlie early neuronal and axonal patterning within the forebrain. The results of this study suggest a novel link between boundaries delineated by gene expression domains and sites of neuronal differentiation and axogenesis. We consistently find that the positions of both neuronal nuclei and the pathways that they pioneer are demarcated by expression boundaries of members of the pax, eph, forkhead, and wnt gene families (summarized in Figure 7).

**Boundaries Demarcate Zones at which Neurons Differentiate**

Although all major early forebrain nuclei differentiate along boundaries defined by gene expression domains, not all gene expression boundaries appear to define sites of neuronal differentiation. However, it remains possible that other expression boundaries are related to later patterns of neuronal differentiation since we only examined early stages of forebrain morphogenesis.

It is striking that the early expression domains of both pax6 and rtk1 define regions within which there is no neuronal differentiation, suggesting that these genes may be acting in a pathway to maintain cells in a proliferative or undifferentiated state. This possibility gains support from the observation that pax6 is heavily expressed in proliferative regions of the eye (R. M. and S. W., unpublished data) and mitotically active cells in the mouse CNS (Walter and Gruss, 1991). It has been proposed that the Xenopus homolog of the Drosophila transmembrane receptor Notch may have a similar, if more widespread, role in maintaining neuroepithelial cells in an undifferentiated state (Coffman et al., 1993).

**Boundaries May Guide Axons**

It has been proposed that pioneering growth cones may be able to detect differences between neuroepi-
Figure 7. Summary Diagrams of Gene Expression Domains and Early Patterning of the Zebrafish Forebrain

The schematic pictures represent brains from approximately 24-26 som embryos.
(A) Prospective regions of the brain.
(B) Pioneer neurons and axon pathways. The diagram does not represent the true size of the neuronal nuclei at this stage, rather it shows the position and approximate numbers of neurons present when the nucleus first differentiates. For clarity, the TPC is included, although this pathway is probably pioneered slightly after the POC and AC have been established.
(C) pax6 (purple) and pax2 (red) expression domains.
(D) axial (red) and wnt1 (purple) expression domains.
(E) rtk1 expression domains (purple).
(F) rtk2 expression domains (red).
(G) shh expression domain (red). This figure is adapted from expression data in Krauss et al. (1993), and exact expression boundaries should be considered tentative.
(H) Summary schematic of boundaries in the developing forebrain and the genes whose expression domains recognize these boundaries. Dashed lines indicate that the genes currently studied do not allow us to accurately define the extent of the boundary. The open circles represent sites of neuronal differentiation. The figure is not exhaustive, and other boundaries almost certainly do exist. ac, position of the anterior commissure; cb, cerebellum; dt, dorsal thalamus; dvdt, dorsoventral diencephalic tract; e, epiphysis; hy, hypothalamus; mlf, medial longitudinal fasciculus; os, optic stalk; p, anterior pituitary; poa, postoptic area; poc, position of the postoptic commissure; pt, pretectum; r1, rhombomere 1; sot, supraoptic tract; t, telencephalon; te, tectum; tg, tegmentum; tpc, tract of the posterior commissure; tpoc, tract of the postoptic commissure; vf, ventral flexure; vt, ventral thalamus.
thelial cells at different locations (Katz et al., 1980). However, in vertebrates, it is largely unknown what these differences might be, and to date there are no reports of any guidance molecule specifically delineating an axon pathway before any axons are present. In this study, we show that boundaries of regulatory gene expression domains are coincident with most of the early axon pathways in the zebrafish forebrain.

We have recently proposed several models of how boundary zones may influence the guidance of pioneering growth cones (Wilson et al., 1993). Perhaps the simplest scenario is one in which a growth cone extends along an interface between two domains of cells with different cell surface properties. Indeed, a potential role for RTK genes is in the regulation of cell surface properties of expressing cells (see, for example, Pulido et al., 1992). The presence of both an immunoglobulin-like loop and fibronectin type III repeats (O'Bryan et al., 1991; Pasquale, 1991) in the extracellular domains of eph family RTKs indicates that these receptors share features with adhesion or recognition molecules that have been implicated in growth cone extension (Hynes and Lander, 1992).

Alternatively, a substrate pathway could be established if boundary cells offer a more favourable environment for growth cone extension than either of the adjacent domains. Analysis of growth cone morphology allows an indirect assessment of the nature of the substrate pathways that are present in the fish brain (Wilson and Easter, 1991b). Even within a single pathway, the TPOC, there are likely to be differences in the extent of the permissive substrate pathway along which growth cones can extend. Near the commissure, the pathway is tightly fasciculated, and growth cones lack both filopodia and lamellipodia, suggesting that neuroepithelial domains neighbouring the pathway are not conducive to growth cone extension or filopodial exploration. Conversely, in the middiencephalon, the TPOC is loosely fasciculated and growth cones are elaborate, suggesting a much broader permissive environment. It is interesting that the position at which the pathway ceases to be tightly fasciculated corresponds to the position at which the strip of cells with ventral character extends into the dorsal diencephalon, a discontinuity in the boundary zone.

It is worth noting that the TPOC is established 1–2 cell diameters distant from the pax6 expression boundary. Perhaps other genes define this pathway more precisely, or alternatively, a boundary zone may exist between forebrain and midbrain, with both the expression boundary and the axon pathway falling within the limits of the boundary tissue. To resolve this and related issues, it will be necessary to determine whether gene expression boundaries in the forebrain define a direct interface between adjacent compartments of cells or alternatively, whether cells at and adjacent to gene expression boundaries develop an identity distinct from nonboundary tissue as has been shown in the hindbrain (see, for example, Heyman et al., 1994).

Regulation of Dorsoventral Patterning in the Forebrain

Although there is much controversy as to whether the anteroposterior patterning of the forebrain shares features with more caudal regions of the CNS (see below and Papalopulu, 1994), it does seem that some aspects of dorsoventral patterning are conserved throughout the entire CNS. For instance, the cyclops mutation affects the specification of ventral midline tissue along the entire length of the CNS (Hatta et al., 1991, 1994). Cell transplantation experiments have suggested that the cyclops gene acts within the neuroepithelium and may be involved in the reception of signals from midline mesoderm (Hatta et al., 1991). The interruption in signaling from prechordal axial mesoderm in cyclops embryos may therefore result in pax6 expression spreading throughout the dorsoventral extent of the forebrain. This interpretation is supported by the finding that when the notochord is removed from beneath the chick neural tube, pax6 expression spreads throughout the ventral cord (Goulding et al., 1993).

Transcription factors of the forhead/HNF3 family and signaling molecules related to Drosophila hedgehog are likely to be involved in the signaling pathway between axial mesoderm and midline neural tissue, as evidenced by their ability to ectopically induce floor plate markers when overexpressed in the neural tube (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1994; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). Moreover, the expression domains of the hedgehog-related gene shh (Krauss et al., 1993) and the forhead-related gene axial are expanded in rostral as compared with caudal CNS, correlating with the expanded area of the forebrain that is disrupted in cyclops mutants. The correlation even holds for the strip of axial- and shh-expressing cells in the middiencephalon, for it is at this location that an abnormal furrow develops in cyclopic embryos. Thus, it appears that the interruption of signals from midline mesoderm in cyclops mutants leads to abnormal regulation of gene expression domains and concomitant alterations in the fate and behavior of ventral forebrain cells.

Analysis of gene expression domains and of the phenotype of cyclops mutants supports the notion that the dorsally directed finger of axial- and shh-expressing cells in the middiencephalon shares features with ventral midline tissue of more caudal regions of the CNS. It is likely that this strip of cells, which we term the middiencephalic boundary, eventually develops into the zona limitans interthalamica described in other species, in that it appears to separate dorsal from ventral thalamus. For instance, the diencephalic expression domains of several distaless-related genes abut the zona limitans interthalamica
in mice and seem to abut the midline of the hindbrain. Using lineage tracing techniques, Figdor and Stern conclude that the chick diencephalon consists of four domains all caudal to the telen- cephalon. In contrast, based upon both morphology and gene expression patterns, Puelles and Rubenstein suggest that six transverse domains exist within the mouse forebrain, three of which subdivide the telen- cephalon and hypothalamus and three of which probably correspond to divisions observed by Figdor and Stern.

Our studies suggest that the forebrain can be divided into a series of domains at stages comparably earlier than those studied by Figdor and Stern or Puelles and Rubenstein. However, at least some of the gene expression domains that we observe can be correlated with subdivisions observed by other authors. For instance, in support of morphological observations in zebrafish (Ross et al., 1992), mice (Puelles and Rubenstein 1993), and chick (Puelles et al., 1987), we suggest that the hypothalamus represents an expansion of the most anteroventral neuroepithelium of the forebrain.

Contrary to Puelles and Rubenstein, we find that gene expression boundaries in the dorso/anterior forebrain are radially organized (see Figure 7H) as opposed to being strictly transverse or longitudinal. However, this is unlikely to reflect a true difference between mice and fish, and comparison of the model of Puelles and Rubenstein with our own indicates that the radial expression domains that we observe may also be present in mice (for instance Dlx-1/2 in Figure 4A of Puelles and Rubenstein, 1993). The difference in interpretation arises from Puelles and Rubenstein describing such domains as a combination of both transverse and longitudinal domains. Further comparative studies will be essential to reconcile differences in interpretation among species. For instance, in the mouse (Boncinelli et al., 1993), otx and em genes may subdivide the anterior forebrain into more domains than we have so far observed, so it will be important to analyze the expression of the fish homologs of these genes with respect to the boundaries that we have proposed.

Both Puelles and Rubenstein and Figdor and Stern describe forebrain domains as segments, whereas we feel that sufficient criteria have not been met to be certain that the subdivisions are segmental. There is no widely accepted definition of segmentation, but a number of characteristics have been used to support a segmental interpretation of hindbrain development. These include morphological segmentation, reiterated patterns of neurogenesis, serial homology of neuronal types, lineage restrictions, and segmental gene expression. Some, though not all, of these features are apparent in the forebrain (Papalopulu, 1994). For instance, Figdor and Stern have shown that lineage restrictions occur at boundaries between domains.

Patterning of Dorso/Anterior Forebrain

Lithium treatment of embryos affects the patterning of dorso/anterior forebrain structures, such as telencephalon and eyes. The early stage at which lithium is given suggests that its effect upon the forebrain may be indirect and that the primary target may be the prechordal mesoderm that eventually underlies the brain. Indeed, other authors have suggested that the lateral migration of goosecoid-expressing prechordal cells is affected by lithium treatment (Stachel et al., 1993). That anterior mesoderm may be necessary for induction of dorso/anterior forebrain is supported by experiments in Xenopus that show that neural tissue lacking underlying prechordal mesoderm fails to develop eyes (reviewed by Ruiz i Altaba and Jessel, 1993).

Forebrain Segmentation

Until recently, the most widely accepted model of forebrain morphogenesis was based on the columnar model proposed by Herrick, Kuhlenbeck, and others (reviewed by Puelles and Rubenstein, 1993). This model proposes that the telencephalon is the most rostral neuromere of the forebrain and that the more caudally positioned diencephalon is divided into four dorsoventral domains. The model fails to take account of the curvature of the axis of the brain and therefore does not adequately define the anteroposterior axis. Thus, when this curvature is taken into account, it appears that the telencephalon is a dorsal evagination and not a discrete neuromere rostral to the diencephalon (Ross et al., 1992; Puelles and Rubenstein, 1993).

Two other models of forebrain morphogenesis have been proposed within the last year (Figdor and Stern, 1993; Puelles and Rubenstein, 1993). Both models suggest that the forebrain can be divided into a series of transverse domains somewhat akin to rhombomeres of the hindbrain. Using lineage tracing techniques, Figdor and Stern conclude that the chick diencephalon consists of four domains all caudal to the telen- cephalon. In contrast, based upon both morphology and gene expression patterns, Puelles and Rubenstein suggest that six transverse domains exist within the mouse forebrain, three of which subdivide the telen- cephalon and hypothalamus and three of which probably correspond to divisions observed by Figdor and Stern.
within the dorsal diencephalon. However, these restrictions appear rather late during forebrain patterning, and it is not clear whether they span the entire dorsoventral extent of the diencephalon. The evidence that the forebrain is patterned very early in development is compelling (Papalopulu, 1994). For example, many genes (including all of those that we examined) are expressed in spatially restricted domains prior to, or from, the end of gastrulation; this is before lineage restrictions would be apparent in the chick. Therefore, it remains possible that the lineage restrictions observed by Figdor and Stern serve to keep cells in different compartments segregated during differentiation rather than to play a role in the primary segmentation of the forebrain neuroepithelium.

In summary, although the forebrain is clearly divided into discrete domains from very early stages of development, these domains do not show simple repetitive patterns of gene expression or neural development as they do in the hindbrain, and as yet, we think that there is insufficient evidence to suggest that each, or alternating, domains in the forebrain derive from a common developmental compartment.

Experimental Procedures

Maintenance of Fish
Embryos were collected by natural spawning, raised at 28.5°C, and staged according to The Zebrafish Book (Westerfield, 1989). cyclops (cyc+) carrier fish were obtained from Charles Kimmel and Stefan Schulte-Mmerker.

Preparation of an Antibody to pax6
The 3' terminal HindIII-EcoRI fragment (596 bp) of the zfpax[a] clone z2K3 (Krauss et al., 1991) encoding the C-terminal part of the protein was cloned into the expression vector pGEX-4X (Amrad Corporation) to give rise to a pax6-GST fusion protein, which was used to generate antibodies as described by Mikolka et al. (1992).

In Vitro Transcription and Translation
Linearized DNA was transcribed in vitro according to the instructions of the manufacturer (Promega). Purified RNA was heated to 67°C for 10 min, and in vitro translation was carried out using the Bovine-R-Mannheim rabbit reticulocyte kit labeling with [35S]methionine. Following translation, samples were stored at -70°C. A 2 μl sample was run on a 16.5% monomer/3% cross-linker polyacrylamide gel to confirm synthesis of pax6 protein. Before immunoprecipitation, 100 μl of TTBS (100 mM Tris-HCl (pH 7.5), 0.5% NaCl, 1% Triton X-100) with protease inhibitors was added to each translation reaction.

Immunoprecipitation of [35S]Methionine-Labeled Products
Immunoprecipitation was carried out according to David et al. (1993), except incubations were performed in TTBS buffer, and beads were washed three times in TTBS and once in TTBS without Triton X-100. Electrophoresis was performed as described above.

Antibody Specificity
The antibody was shown to recognize pax6 protein through its ability to immunoprecipitate an in vitro translated and translated pax6 protein of an expected molecular weight of 48 kDa (Figure 8). Specificity was confirmed in that the antibody revealed identical expression domains, as was seen by in situ hybridization.

Whole-Mount In Situ Hybridization
Methods for combined antibody labeling and in situ hybridization and preparation of rtk RNA probes were as described in Xu et al. (1994). Antisense digoxigenin-labeled RNA probes were synthesized from the full length pax6 cDNA clone z2K3 (Krauss et al., 1991b, 1991c), a 1740 bp axial cDNA clone (Strähle et al., 1993), and a 650 bp genomic wnt7 clone (Molven et al., 1991). All probes were partially hydrolyzed under alkaline conditions to an average size of 400-500 nt.

Antibody Protocols, Dil Labeling, and Sectioning
Procedures for antibody, Dil labeling, and sectioning protocols are as described in The Zebrafish Book (Westerfield, 1989). For primary incubations, anti-pax6 antibody was diluted 1:400; anti-pax2 antibody (Mikolka et al., 1992), 1:3000; anti-acetylated tubulin (anti-AI), 1:20; anti-CRAB (Chemicon), 1:2000. The antibodies that we used to reveal neurons (HNK1 and anti-AI) only label cells that have initiated axogenesis; however, it is likely that zones of neuronal differentiation colocalize with sites of neurogenesis. For instance, acetylcholinesterase is present in neurons soon after their final mitoses (Layer, 1983), and acetylcholinesterase activity colocalizes with differentiating neurons (Wilson et al., 1990; Ross et al., 1992).

Lithium Treatment of Embryos
Embryos at the sphere stage were immersed in their chorions for 8–11 min in 0.3 M LiCl at 28.5°C, as described in Stachel et al. (1993), and were fixed between 24 and 30 hr of development.

Nomenclature
The pax6 and pax2 genes were originally published as zfpax[a]