Neuron, Vol. 14, 1141–1152, June, 1995, Copyright © 1995 by Cell Press

Winged Helix Transcription Factor BF-1 Is Essential for the Development of the Cerebral Hemispheres

Shouhong Xuan,*‡ Carlos A. Baptista,*‡ Gabriela Balas,* Wufan Tao,* Vera C. Soares,† and Eseng Lai*
*Cell Biology and Genetics Program
Division of Endocrinology
†Molecular Biology Program
Sloan-Kettering Division
Cornell University Graduate School of Medical Sciences
Memorial Sloan-Kettering Cancer Center
New York, New York 10021

Summary

We generated mice with a null mutation of the forebrain-restricted transcription factor BF-1 to examine its function in brain development. Heterozygous animals have an apparently normal phenotype. Homozygous null BF-1 mutants die at birth and have a dramatic reduction in the size of the cerebral hemispheres. The development of the ventral telencephalon is more severely affected than that of the dorsal telencephalon. Telencephalic neuroepithelial cells are specified in the BF-1 mutant, but their proliferation is reduced. Dorsal telencephalic neuroepithelial cells also differentiate prematurely, leading to early depletion of the progenitor population. These results suggest that BF-1 controls the morphogenesis of the telencephalon by regulating the rate of neuroepithelial cell proliferation and the timing of neuronal differentiation.

Introduction

The capacity of the mammalian brain to perform complex tasks depends on the activity of a large number of neurons and neuronal cell types that are organized into numerous regions specialized for distinct functions. All of the cells of the CNS arise from the neuroepithelium, a morphologically uniform sheet of cells that folds to form the neural tube. Patterning of the neuroepithelium along the anteroposterior axis leads to the progressive subdivision of the neural tube into regions with distinct fates (Keynes and Lumsden, 1990). Families of regulatory genes with restricted patterns of expression within the neuroepithelium have been identified that are believed to function to establish positional identity in the neuroepithelium and to control region-specific morphogenesis of the brain (Krumlauf et al., 1993; Figdor and Stern, 1993; Puelles and Rubenstein, 1993). The telencephalon gives rise to the structures of the cerebral hemispheres, which include the cerebral cortex and the basal ganglia. While several regulatory gene families have been recently described with expression boundaries within the telencephalic neuroepithelium, including *Otx*, *Emx*, *Dlx*, and *Pax* genes (Simeone et al., 1992; Porteus et al., 1991; Price et al., 1991; Chalepakis et al., 1993), the mechanisms controlling the development of this largest and most complex region of the brain remain the least well understood. One of the striking features of mammalian telencephalon development is its increased and prolonged growth relative to the other regions of the neural tube. This relative expansion of the cerebral hemispheres is an important component of vertebrate evolution. Encephalization of the brain is most prominent in mammals, particularly in primates, and is associated with the acquisition of higher cognitive functions (Hofman, 1989). Very little is presently known about the molecular mechanisms that control either the specification or the growth of the telencephalon.

We have previously identified brain factors 1 and 2 (BF-1 and BF-2, respectively), two new members of the winged helix (WH) family of transcription factors. The members of this gene family share a DNA binding domain, first identified in the HNF-3 proteins (Lai et al., 1990, 1991). This domain has been called the winged helix based on its three-dimensional structure determined by X-ray diffraction of an HNF-3γ/DNA cocrystal (Clark et al., 1993). Many WH genes have been shown to play critical roles during development (reviewed in Lai et al., 1993). BF-1 expression in the developing brain is restricted to the telencephalic neuroepithelium and the nasal half of the retina and optic stalk. Its expression domain is adjacent to that of BF-2, which is restricted to the rostral diencephalon and the temporal half of the retina and optic stalk (Tao and Lai, 1992; Hatini et al., 1994). BF-1 is detected in the neuroepithelium prior to the morphological appearance of the telencephalon–diencephalon boundary, suggesting a role in patterning the forebrain. Expression levels of BF-1 are highest in the rapidly proliferating cells of the neuroepithelium, declining as the cells become postmitotic. Further evidence for a role for BF-1 in growth control is suggested by the identification of an oncogene from an avian sarcoma virus, *qin*, as the chicken homolog of BF-1 (Li and Vogt, 1993). From these observations, we postulated that BF-1 may function to specify the positional identity of the telencephalic neuroepithelium and to determine its proliferative potential during brain development (Hatini et al., 1994).

To examine the function of BF-1 in brain development, we generated mice with a null mutation in the BF-1 gene. Deletion of both copies of BF-1 leads to a marked reduction in the size of the cerebral hemispheres. The growth of the ventral telencephalon is more severely affected than that of the dorsal telencephalon. We demonstrate a reduction in the proliferation rate of the dorsal and ventral telencephalic neuroepithelium as well as alterations in the timing of neuronal differentiation in the cerebral cortical neuroepithelium. These findings establish an essential role for BF-1 in forebrain development and suggest that it may function in the developmental control of cell proliferation and neuronal differentiation.

‡These authors contributed equally to this work.

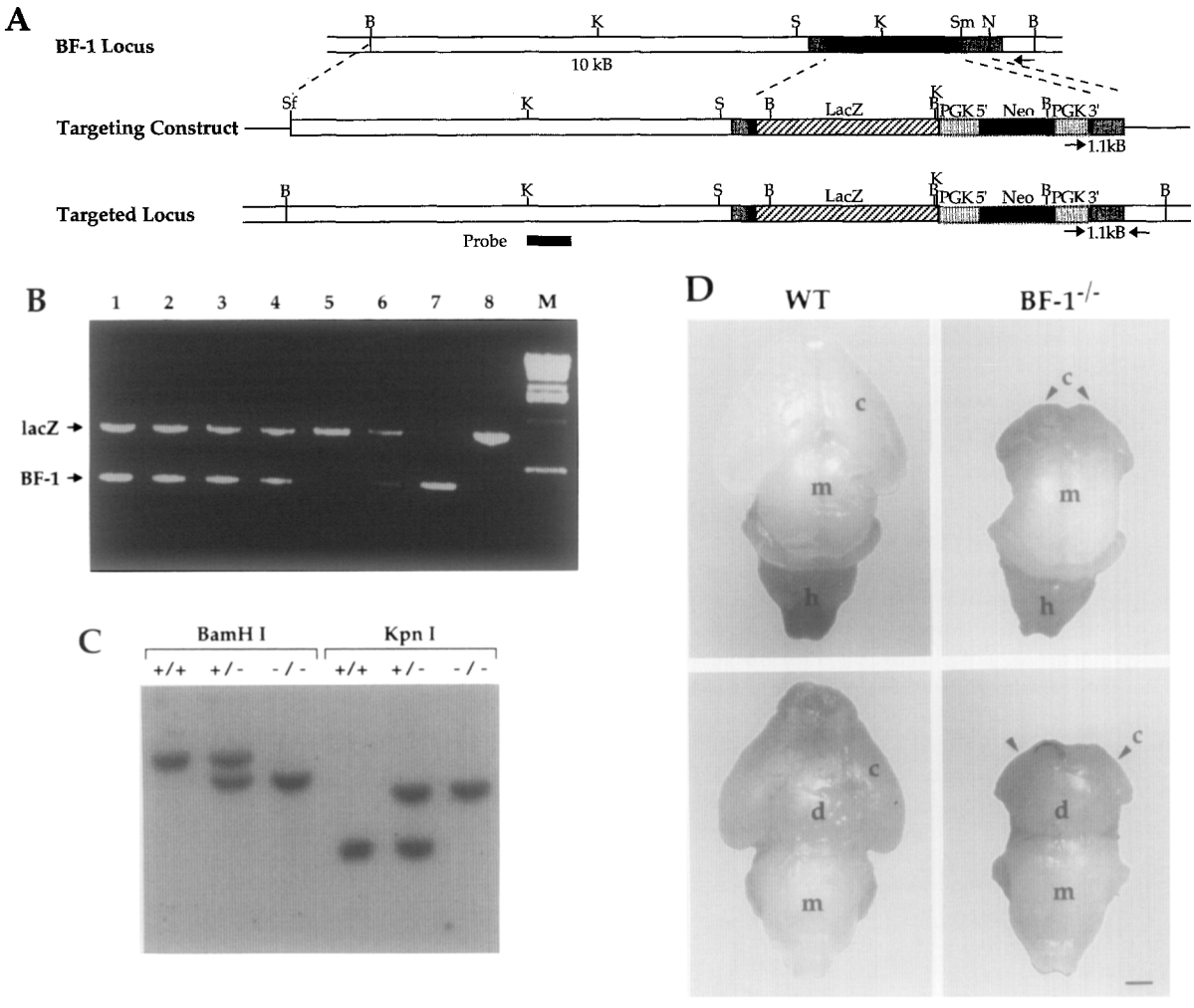

Figure 1. Targeted Disruption of the BF-1 Gene and Resulting Brain Phenotype

(A) Targeting vector. Nearly all of the coding sequence of BF-1 was deleted by removal of a 2.3 kb fragment in the genomic DNA. The *lacZ* sequence with a nuclear localization signal was fused in-frame with the first 13 amino acids of BF-1 to express functional β-galactosidase (β-gal) under the control of the BF-1 promoter. B, BamHI; K, KpnI; N, NdeI; S, SalI; Sf, SfiI; Sm, SmaI.

(B) PCR analysis of a typical litter to genotype the offspring of heterozygote matings. Two sets of primers were used to identify the presence of lacZ (822 bp band) or the BF-1 sequences (295 bp band) in genomic DNA from a typical litter. The absence of a PCR product using the BF-1 primers confirms that these sequences are deleted in the BF-1 homozygote.

(C) Southern analysis of littermates from a heterozygote mating to confirm the genotype of animals identified initially by PCR. The probe shown in (A) hybridizes to genomic fragments generated by the restriction endonucleases BamHI (11 and 14 kb) and KpnI (6 and 9 kb).

(D) Brains dissected from newborn wild-type (WT) and BF-1 mutant (BF-$1^{-/-}$) littermates: dorsal (upper pair) and ventral (lower pair) views. The mutant brain has markedly reduced cerebral hemispheres (arrowheads) that rest on the dorsolateral surface of the diencephalon. The remainder of the brain appears normal in the mutant. c, cerebral hemisphere; d, diencephalon; h, hindbrain; m, midbrain. Bar, 1 mm.

Results

Generation of a Null Mutation in BF-1

A null mutation of BF-1 was generated by replacing most of the coding sequence of BF-1, which is contained in a single exon, with a lacZ and neomycin cassette (Figure 1A). The β-galactosidase (β-gal) sequence containing a nuclear localization signal was fused in-frame to the first 13 amino acids of BF-1, with the goal of placing the expression of this enzyme under the control of the BF-1 promoter and the BF-1 translation initiation site. Electroporated embryonic stem (ES) cells (CJ7 and J1 lines) integrating the targeting construct were selected for drug resistance with G418. Cells undergoing homologous recombination were identified initially by the polymerase chain reaction (PCR) and confirmed by genomic Southern analysis (Figures 1B and 1C). Correctly targeted ES cells were injected into C57BL/6J host blastocysts to generate chimeric mice. Chimeric males from 1 clone each of ES cells from CJ7 and J1 lines transmitted the targeted DNA through the germline, generating mice that were heterozygous for the BF-1 deletion. Heterozygotes are fertile and indistinguishable from wild-type littermates, except by the expression of β-gal. Matings between heterozygotes yielded live homozygous offspring when pregnant mothers were observed during delivery (Table 1). These newborn animals were flaccid

Table 1. Genotype of Offspring from BF-1 (+/−) Heterozygote Matings

Age	Litters	Animals	Genotype (+/+)	(+/−)	(−/−)
E8.5	1	7	1	3	3
E9.5	7	50	9	26	15
E10.5	12	59	23	56	18
E11.5	5	45	8	25	12
E12.5	20	112	41	63	31
E14.5	1	9	2	4	3
E16.5	2	10	2	4	4
E18.5	4	30	11	9	10
P0	7	53	15	27	11
Total (E8.5–birth)	59	436	112 (26%)	217 (50%)	107 (24%)
>P1	5	36	11 (31%)	25 (69%)	0 (0%)

Homozygous BF-1 mutants survive to term. P0 indicates litters for which delivery was observed. All of the homozygous mutants were born alive, but they all died within 20 min of birth.

and displayed minimal spontaneous movement except for intermittent gasping motions. All of them remained cyanotic and died within minutes after birth. At autopsy, the alveoli of the lungs were uninflated (data not shown), demonstrating that death was due to respiratory failure, although the exact cause remains unclear. Homozygous BF-1 mutant animals derived from targeted CJ7 and J1 ES cells have the same phenotype. The studies presented are from CJ7-derived animals.

BF-1 (−/−) Mutants Have Small Cerebral Hemispheres

Newborn BF-1 (−/−) mutants were distinguishable from wild-type and heterozygous littermates by a flattening of the frontal skull and by abnormalities of the eyes. Examination of the newborn brain showed that the cerebral hemispheres were reduced by 95% in mass (Figure 1D). While the cerebral hemispheres normally grow to surround the diencephalon, the diminutive mutant cerebral hemispheres of the BF-1 mutant rest on the dorsolateral surface

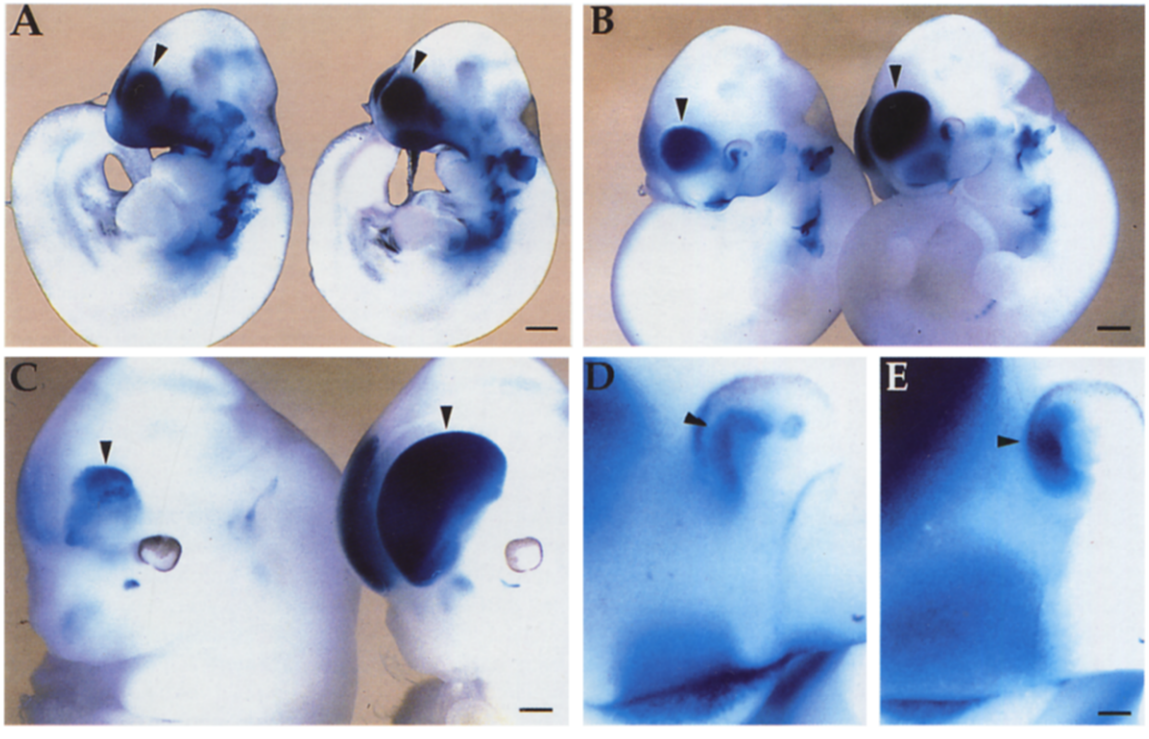

Figure 2. Expression of β-gal in Whole-Mount Embryos Reveals a Progressive Reduction in the Size of the Telencephalic Vesicles and Abnormalities of the Eyes

Detection of β-gal was done by X-Gal histochemistry. Pairs of embryos are shown with the mutant (−/−) on the left and the heterozygote (+/−) on the right at E9.5 (A), E10.5 (B), and E12.5 (C). High power views of the eye regions in the mutant (D) and heterozygous (E) embryos shown in (B) are also shown. The β-gal staining identifies structures that normally express BF-1. In (A)–(C), arrowheads point to the telencephalic vesicles of each embryo. While at E9.5 there are only minimal differences in the size of the telencephalic vesicles between the mutant and heterozygote (A), the growth of the vesicles in the mutant is progressively retarded relative to the heterozygote at E10.5 (B) and E12.5 (C). The surface of the telencephalic vesicles is also irregular in the E12.5 mutant. Prominent in the mutant eye is the wavy appearance of the anterior retinal neuroepithelium (D and E, arrowheads) as well as the ventral rotation of the entire eye. Bars, 200 μm (A), 400 μm (B and C), 100 μm (D and E).

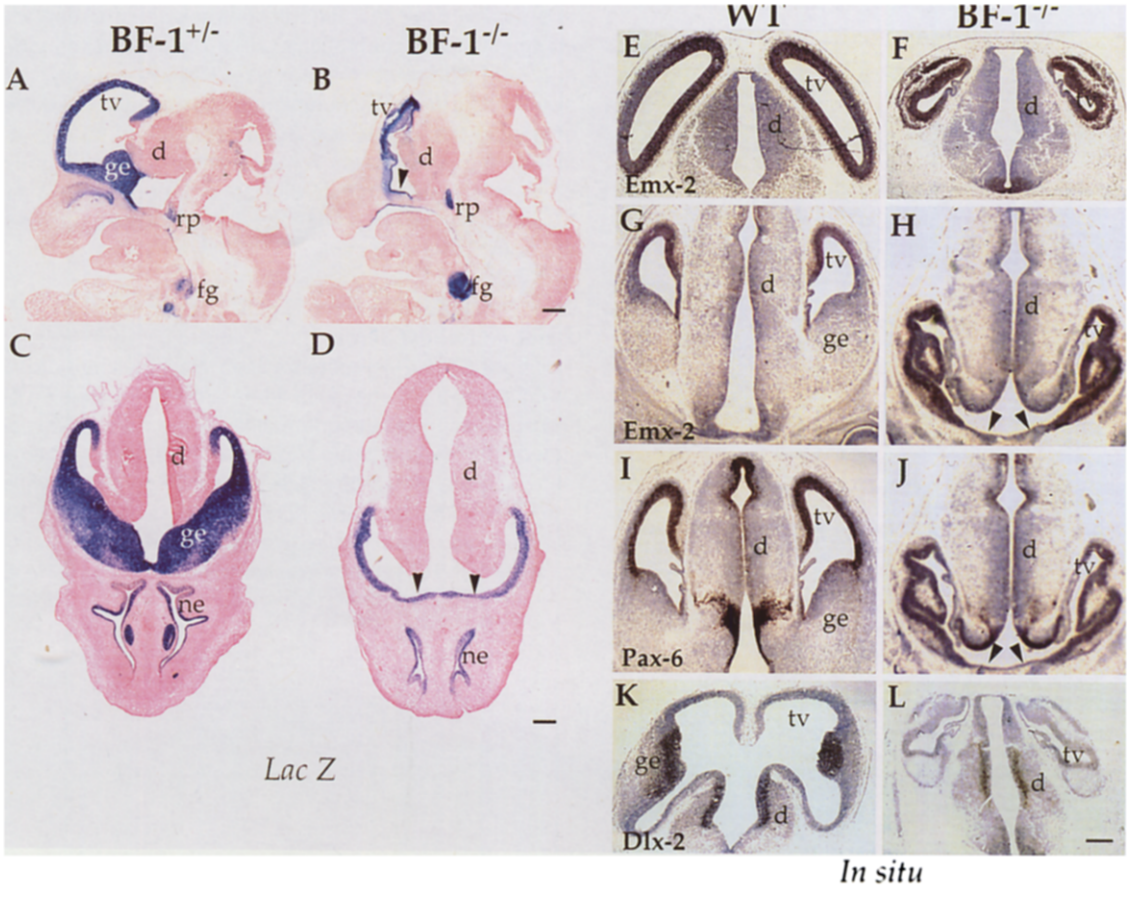

Figure 3. Development of the Ventral Telencephalon Is More Severely Affected Than That of the Dorsal Telencephalon in the BF-1 Mutant

Expression of β-gal identifies structures derived from the telencephalon in E12.5 BF-1 (+/–) heterozygotes and BF-1 (–/–) mutants ([A and B] sagittal views; [C and D] coronal views). Expression patterns of markers for the dorsal telencephalon, Emx2 (E–H) and Pax6 (I and J), and for the ventral telencephalon, Dlx2 (K and L), were analyzed by in situ hybridization in wild-type (WT) and mutant (BF-1$^{-/-}$) E12.5 embryos. Sections were matched to show comparable structures in each pair. Horizontal views show the expression of Emx2 in the telencephalic vesicles of both wild-type (E) and mutant (F) embryos. Coronal sections at a level anterior to the eyes show the telencephalic vesicles and the ganglionic eminences in wild-type embryos (G and I). Both Emx2 and Pax6 are restricted to structures derived from the dorsal telencephalon. The mutant dorsal telencephalon extends more ventrally and medially than normal, as demonstrated by the expression pattern of both Emx2 (H) and Pax6 (J). The small region of the ventral telencephalic neuroepithelium (indicated by arrowheads) that does not express either dorsal or ventral markers most likely represents vestigial ventral telencephalic neuroepithelium. Horizontal views at a more ventral level than those in (E) and (F) show the ganglionic eminences in wild-type embryos (K), which, along with the diencephalic neuroepithelium, express Dlx2. Expression of Dlx2 in the mutant is confined to the diencephalon (L). Embryos are from two litters, nominally at E12.5; however those in (A)–(D) are smaller and younger than those in (E)–(L). d, diencephalon; fg, foregut; ge, ganglionic eminence; ne, nasal epithelium; rp, Rathke's pouch; tv, telencephalic vesicles. Bars, 400 μm (A and B), 200 μm (C–L).

of the diencephalon. The olfactory bulbs were also markedly reduced in size. The remainder of the brain appeared normal by gross inspection in the newborn and by light microscopy in midgestation embryos (E10.5–E14.5). Whole-mount heterozygous embryos stained for β-gal activity showed that the staining pattern matched closely the normal pattern of BF-1 RNA expression detected by in situ hybridization. β-gal expression in the BF-1 (–/–) homozygous mutant was also detected in the same tissues, which permitted us to identify tissues in which BF-1 would have been expressed if the gene had not been deleted (Figure 2). BF-1 is first detected in the neural tube between E8.5 and E9.0. At E9.5, heterozygous and mutant embryos displayed minimal differences in the size of the telencephalic vesicles, which have just begun to form (Figure 2A). Differences in the size of the vesicles at this age are associated with the small variation in the developmental stage of embryos within a litter rather than with a clear abnormality in the BF-1 mutant. By E10.5, however, the telencephalic vesicles in the homozygous mutant were distinctly smaller than those in heterozygotes (Figure 2B). A further reduction in the size of the telencephalic vesicles of the mutant relative to the heterozygote was apparent in E12.5 embryos (Figure 2C). In addition to the small size, the normal uniform and smooth appearance of the developing cerebral cortex was replaced in the BF-1 mutant with an irregular, uneven appearance. This was due both to buckling of the neuroepithelium and to variations in the thickness of the cortical tissue (see below). Thus, the telencephalon initially appeared to be formed normally in the BF-1 mu-

tant. Differences between mutant and wild-type embryos became progressively more apparent as development proceeded.

The eyes of newborn BF-1 (−/−) mutant animals were distorted in shape, being ellipsoid instead of round. Irregularities in the contour of the retina were apparent, most prominently in the nasal retina (compare Figures 2D and 2E). The development of nasal structures arising from the nasal placode was also affected in the BF-1 mutant. While the nasal placodes of the heterozygote and mutant homozygote are comparable at E9.5 (Figure 2A), the mutant nasal placodes and the nasal epithelium arising from them fail to grow normally (Figures 2B–2E; Figure 3D). Further studies are in progress to examine the mechanisms that underlie these developmental defects. In contrast, the structures arising from the otic vesicle, pharyngeal pouches and Rathke's pouch (e.g., the inner ear, the anterior foregut, and the pituitary), were not appreciably affected in the BF-1 mutant (Figure 2; Figure 3; and data not shown). Additional analyses will be required to determine whether there are subtle anomalies of these structures in the BF-1 mutant.

Failure of the Ventral Telencephalon to Grow in BF-1 (−/−) Mutants

Further examination of the brain of the BF-1 (−/−) mutant revealed that the development of the ventral telencephalon is more severely affected than that of the dorsal telencephalon. The cerebral cortex is the major structure to develop from the dorsal telencephalon, while most of the ventral telencephalon will give rise to the ganglionic eminences that are the primordia of the basal ganglia. Normally, the ventral telencephalon expands dramatically between E9.5 and E12.5. During this period, ventral telencephalic neuroepithelial cells proliferate rapidly and begin to differentiate. Postmitotic neurons migrate outward from the ventricular zone to form the ganglionic eminences. The expansion of the ventral telencephalon normally displaces the telencephalic vesicles laterally and dorsally (Figures 3A, 3C, 3G, 3I, and 3K). At E12.5 in the BF-1 mutant, the ganglionic eminences were not present (Figures 3B, 3D, 3H, and 3J). Sagittal and coronal views showed only a thin neuroepithelium in their place (Figure 3, arrowheads), which expressed β-gal thus confirming its telencephalic origin (Figure 3D). The dorsal telencephalic vesicles of the BF-1 mutant are positioned more ventrally and closer to the level of the eyes than the corresponding structures of wild-type embryos. These observations suggest that, while the dorsal telencephalon of the BF-1 mutant is reduced in size, the ventral telencephalon is almost completely absent.

In situ hybridization studies examining the expression patterns of the dorsal telencephalic markers Emx1, Emx2 (Simeone et al., 1993), and Pax6 (Walther and Gruss, 1991) as well as the ventral telencephalic markers Dlx1 and Dlx2 (Porteus et al., 1991; Price et al., 1991) further support this conclusion. We found that almost all of the telencephalic neuroepithelium at E12.5 in the BF-1 mutant expressed the dorsal markers Emx2 (Figures 3F and 3H), Pax6 (Figure 3J), and Emx1 (data not shown). Neither ventral marker, Dlx2 (Figure 3L) and Dlx1 (data not shown), was detectable in these tissues. In younger embryos, the thicknesses of the dorsal and ventral regions of the neuroepithelium were more comparable than at E12.5. At E10.5, the expression domain of dorsal markers also extended more ventrally in the mutant (Figures 4C and 4D), while ventral markers were not detected (data not shown). Thus, most of the small, distorted telencephalic tissues found in the BF-1 mutant embryos are dorsal structures representing primarily the developing cerebral cortex. The tissue that expressed the β-gal marker (Figures 3B and 3D, arrowheads) but was negative for the expression of the dorsal telencephalic markers, Emx2 (Figure 3H; Figure 4D) and Pax6 (Figure 3J), we believe to be the vestigial ventral telencephalic neuroepithelium. However, because we did not detect the expression of Dlx1 or Dlx2 in these cells (Figure 3L), it remains possible that ventral specification is abnormal in these nondorsal telencephalic cells.

Reduced Proliferation of the Telencephalic Neuroepithelium in BF-1 (−/−) Mutants

Failure of the telencephalon to grow normally in the BF-1 mutant could be due to several mechanisms, including a reduction in the rate of proliferation of the neuroepithelial precursors, a reduction in the fraction of cells that continue to divide, or an increase in the rate of cell death or apoptosis. No significant differences in apoptosis, as determined by the number of pyknotic nuclei, were observed in the forebrain of BF-1 homozygous mutant or wild-type embryos (data not shown). This suggested that increased cell death is unlikely to be the primary reason for the small size of the telencephalon in the BF-1 mutant. We therefore studied the effect of the BF-1 deletion on the proliferation and differentiation of the neuroepithelial cells of the telencephalon. Cell proliferation in the neuroepithelium was examined by bromodeoxyuridine (BrdU) labeling. BrdU is incorporated into cells in S phase. The fraction of cells labeled with BrdU during a short pulse reflects the fraction of cells in S phase during this period, permitting an assessment of the proliferation rate of a population of cells (Gratzner, 1982). No significant differences were observed in the BrdU labeling patterns of mutant and wild-type embryos at E9.5 (data not shown). Differences in BrdU labeling of the telencephalic neuroepithelium become apparent only later in development, corresponding to the progressively greater differences in the size of the telencephalon between mutant and wild-type embryos after E9.5. In coronal sections of E10.5, we used lacZ expression to identify the telencephalon (data not shown) and Emx2 as a marker of dorsal telencephalon (Figures 4C and 4D). The identity of the ventral telencephalon was also confirmed morphologically in wild-type embryos by the increased thickness of the developing ganglionic eminences (Figures 4A and 4C, arrowheads). The fraction of BrdU-labeled cells was uniformly high in both dorsal and ventral regions of the wild-type telencephalon (Figures 4A and 4E). BrdU labeling in the E10.5 mutant revealed a clear difference between the dorsal and ventral regions of the telencephalon.

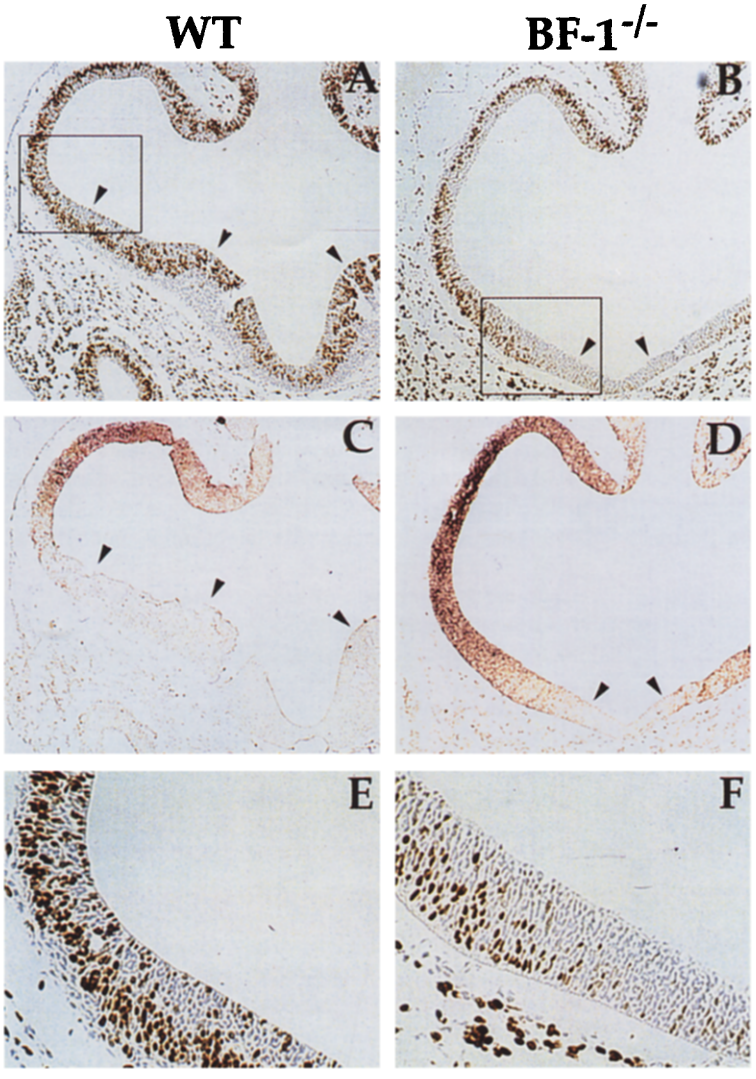

Figure 4. Reduced Cell Proliferation in the Ventral Telencephalic Neuroepithelium of the BF-1 Mutant

Proliferative activity in the telencephalic neuroepithelium of E10.5 wild-type (WT) and mutant (BF-1$^{-/-}$) animals was detected by BrdU immunohistochemistry (A, B, E, and F). All sections are in the coronal plane just anterior to the optic vesicles. Adjacent sections were processed for in situ hybridization with a dorsal marker, Emx2, to define the boundary between dorsal and ventral telencephalon (C and D). The dorsal telencephalon as marked by Emx2 is positioned more lateral and ventral in the mutant. (E) and (F) show high power views of the dorsal–ventral boundary region delineated in (A) and (B), respectively. Arrowheads indicate the ventral telencephalon. Bar, 120 μm (A–D), 40 μm (E and F).

Quantitation of these results is presented in Table 2. These data show a 79% reduction in the proliferative activity of the precursor cells in this region. Thus, in the E10.5 BF-1 mutant, only the dorsal telencephalic neuroepithelial cells, which express Emx2, are actively proliferating. These results suggest that the absence of the ventral telencephalon in the BF-1 mutant is due primarily to the failure of the precursor cells in this region to continue proliferating actively after E9.5.

In the dorsal telencephalic neuroepithelium, the differences in the BrdU labeling pattern between wild-type and BF-1 mutant embryos were more subtle and became apparent later in development. The fraction of BrdU-labeled cells throughout the telencephalic neuroepithelium was uniform in wild-type embryos at E12.5 (Figure 5A). In BF-1 mutants at E12.5, there were significant variations in the fraction of BrdU-labeled cells within the dorsal telencephalic neuroepithelium, most notably a 2-fold difference between anterior and posterior regions (Figure 5B; Table 2). Thus, at least part of the reduction in the growth of the dorsal telencephalon in the BF-1 mutant is attributable to areas of reduced proliferation within the neuroepithelium. We also observe a buckling of the ventricular surface at the boundary between areas with different fractions of BrdU-labeled cells, and therefore different rates of cell proliferation (Figure 5B). This suggests that some of the irregularities in the cerebral cortex of the BF-1 mutant arise from the nonuniform rates of cell proliferation within the neuroepithelium. These studies do not distinguish between a lengthening of the cell cycle and an increase in the fraction of the cells that have left the cell cycle as the basis for the decreased rate of cell proliferation.

The similar morphology and the comparable BrdU labeling patterns of mutant, heterozygous, and wild-type embryos at E9.5 suggest that cell proliferation in the telencephalic neuroepithelium is initially independent of BF-1. However, the proliferation of the ventral telencephalic neuroepithelium in the BF-1 mutant is reduced progressively such that this region stops growing by E10.5. Abnormalities in the proliferation of the dorsal telencephalic neuroepithelium also become more severe as development proceeds.

Table 2. BrdU Labeling in the Telencephalic Neuroepithelium

	Percentage of BrdU Labeling ± SEM	
	Wild Type	BF-1 (−/−)
E10.5 coronal section		
Dorsal	45 ± 2	42 ± 2
Ventral	41 ± 1	9 ± 1
E12.5 horizontal section		
Anterior	43 ± 2	25 ± 8
Posterior	47 ± 3	46 ± 3

Embryos were pulse labeled with BrdU and stained by immunohistochemistry as described in Experimental Procedures. Nuclei were counted under a Zeiss Axioplan light microscope equipped with a reticular eyepiece, in units of neuroepithelium 100 μm in length. Representative areas demonstrating differences in the BF-1 mutant were chosen for counting, as were comparable areas in the wild type. The fraction of BrdU-labeled cells was determined by dividing the number of positive nuclei (brown) by the total number of nuclei. Groups of 2–3 consecutive tissue sections from the same animals were used. For the dorsal–ventral differences at E10.5, wild-type data were pooled from a total of 18 sections from three embryos, while the mutant data was pooled from a total of 22 sections from four embryos. For the anterior–posterior differences found in E12.5 dorsal telencephalon, both wild-type and mutant data were obtained from 9 sections from three embryos. Data are expressed as the mean ± SEM.

Premature Onset of Neuronal Differentiation in BF-1 (−/−) Mutants

The major period of cerebral cortical neuronal differentiation in the mouse occurs between E11 and E18 (Caviness and Sidman, 1973). The neuroepithelium or the ventricular zone is comprised of mitotically active progenitor cells and is the source of all neurons and glia in the adult cerebral cortex. Prior to E11, essentially all of the neuroepithelial cells are actively dividing. As development progresses, an increasing proportion of the neuroepithelial cells start to exit the cell cycle, begin to differentiate, and migrate away from the ventricular zone to populate the layers of the cortex (Angevine and Sidman, 1961; Berry et al., 1964). At the beginning of this period of neurogenesis, the neuroepithelium comprises nearly the entire thickness of the cerebral cortex. Only the preplate neurons, which are the earliest to be born, are present on the outer surface of the ventricular zone (Marin-Padilla, 1990). Gradually, the thickness of the cortical plate increases as more cells leave the neuroepithelium and migrate outward.

The cerebral cortex of the BF-1 mutant was observed to vary in thickness compared with the uniform appearance of the wild-type cerebral cortex. By clearly delineat-

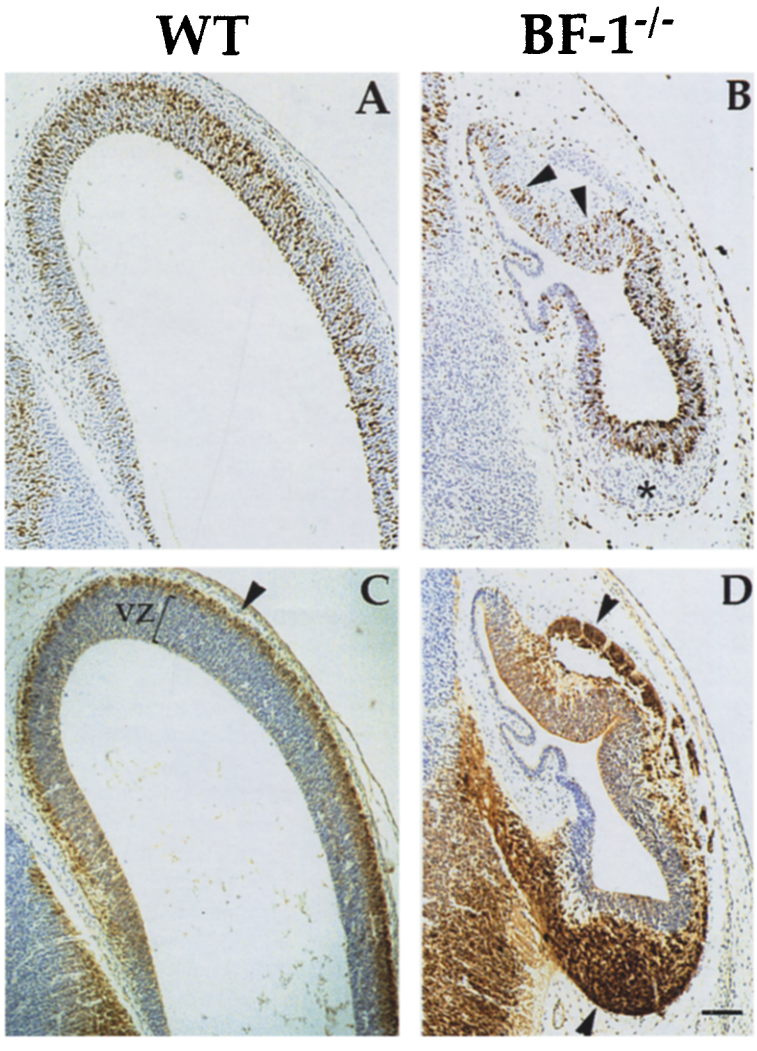

Figure 5. Reduced Cell Proliferation in the Dorsal Telencephalic Neuroepithelium Is Accompanied by Early Onset of Neuronal Differentiation in the BF-1 Mutant

Adjacent horizontal sections at the level of the neocortex in wild-type animals were processed for immunodetection of BrdU (A and B) and MAP2 (C and D) to determine the proliferative activity and the pattern of neuronal differentiation, respectively, in E12.5 animals. The ratio of BrdU-labeled cells is uniform in the wild-type (WT) neuroepithelium (A), whereas in the mutant (BF-1$^{-/-}$), there is an anterior region of low proliferation (arrowheads) adjacent to a posterior region with a higher fraction of labeling (B). Note the buckling of the surface of the neuroepithelium at the boundary between these two regions. In the wild-type cerebral cortex, the early differentiating neurons, as demonstrated by MAP2 labeling, are restricted to the narrow preplate layer (C, arrowhead). In the mutant, cells outside of the ventricular zone (asterisk) are greatly increased in proportion to those within it. These cells are present both anteriorly and posteriorly and are MAP2-positive (D, arrowheads). vz, ventricular zone. Bar, 100 μm.

ing the neuroepithelium, BrdU labeling also revealed that this was due to an increase in the fraction of cells outside the ventricular zone. Whereas the ventricular zone comprised nearly all of the cerebral cortical tissue in wild-type E12.5 embryos, it appeared to make up a smaller fraction of the cortical tissue in the BF-1 mutant. In some areas of the mutant cerebral cortex at E12.5, the ventricular zone represented less than one-quarter of the thickness of the cortical tissue. This difference was due mainly to an increase in the number of cells outside of the ventricular zone (Figure 5B, asterisk). To determine whether these cells were differentiating neurons, we used a marker of early postmitotic neurons, microtubule-associated protein 2 (MAP2; Crandall et al., 1986), and compared its expression in wild-type and mutant embryos.

In normal cerebral cortex at E12.5, MAP2 expression was detected only in the preplate neurons (Figure 5C). The increased fraction of cells in the BF-1 mutant embryo that were outside of the ventricular zone all expressed MAP2 (Figure 5D). These MAP2-positive cells did not label with BrdU, suggesting that they were postmitotic cells committed to neuronal differentiation. The expression of the dorsal markers Emx1 and Emx2 in these structures argues against the possibility that the BF-1 mutation causes a transformation of the dorsal telencephalon to a more ventral fate. Rather, the data suggest that the BF-1 deletion results in the early withdrawal of telencephalic neuroepithelial cells from the cell cycle and in the premature onset of neuronal differentiation. Because most of these postmitotic cells migrate away from the ventricular zone, the fraction of BrdU-labeled cells in the neuroepithelium remained unchanged, even though the fraction of cells apparently exiting the cell cycle increased. This is most obvious in the posterior region of the BF-1 mutant cerebral cortical neuroepithelium, where there was extensive expression of MAP2 (Figures 5B and 5D). We suggest that these events result in the depletion of progenitor cells at an earlier stage in development, leading to a smaller number of cells that are ultimately generated in the dorsal telencephalon.

Discussion

BF-1 and the Development of the Cerebral Hemispheres

BF-1 function is required for the normal development of the telencephalon. Two features of the BF-1 null mutant phenotype provide unique insights into the mechanisms controlling the regional subdivision of the forebrain and the differential growth of regions within the telencephalon. In prior analyses of targeted deletions that result in developmental anomalies of the brain, it has often been difficult to distinguish between defects in cell specification and cell proliferation (Thomas and Capecchi, 1990; Joyner et al., 1991; McMahon et al., 1992). We are able to address this issue in the BF-1 mutant because of both the β-gal expression marker and the partial development of the cerebral hemispheres. The telencephalic neuroepithelium in both BF-1 heterozygotes and mutant homozygotes can be identified by the expression of β-gal activity and by its morphology in early embryos. At E9.5, minimal differences in the telencephalic neuroepithelium are apparent between heterozygotes and mutants. As development progresses, tissues in which the BF-1 promoter is active remain clearly demarcated in both the heterozygote and the mutant, despite differences in the size of these structures. Even the ventral region that fails to grow can be identified as telencephalic neuroepithelium in the early mutant embryo by morphology and β-gal staining. These results suggest that specification of the telencephalic neuroepithelium does not require BF-1. The mechanisms that initially subdivide the forebrain neuroepithelium remain functional in the BF-1 mutant. However, BF-1 is essential for the normal growth and differentiation of the telencephalic neuroepithelium after its positional identity is determined.

We next examined the mechanism by which BF-1 controls the growth of the telencephalic neuroepithelium. At E9.5, the fraction of cells labeled with a pulse of BrdU in the telencephalon of the BF-1 mutant is indistinguishable from that of normal embryos. However, by E10.5, we observe a dramatic reduction in BrdU-labeled cells in the ventral telencephalic neuroepithelium of the mutant. Together with the β-gal expression results, these data suggest that, although telencephalic neuroepithelial cells in the BF-1 mutant are initially able to proliferate after they are specified, abnormalities in cell proliferation develop soon thereafter. We conclude that BF-1 is essential for the continued proliferation of the ventral telencephalic neuroepithelium after E9.5. The mutant dorsal telencephalic neuroepithelium appears to proliferate normally for a longer period of time; however, by E12.5 we observe areas of diminished BrdU labeling interspersed with areas of apparently normal fractions of labeled cells. We also find an abnormally wide zone of cells outside of the cerebral cortical ventricular zone that expresses MAP2 and does not label with BrdU, suggesting that cells of the dorsal telencephalic neuroepithelium are prematurely leaving the cell cycle and beginning to differentiate earlier than usual. Thus, proliferation of the dorsal telencephalic neuroepithelium is altered in the BF-1 mutant, but at a later developmental stage than in the ventral neuroepithelium. We propose that the resulting early depletion of the progenitor cell population in the dorsal telencephalic neuroepithelium is the primary basis for the small size of the dorsal telencephalon in the BF-1 mutant.

The nonuniform consequences of the BF-1 deletion on the growth and development of the telencephalon might seem to be inconsistent with the uniform expression of BF-1 in the telencephalon. Whereas the ventral telencephalon fails to grow appreciably after E9.5, the dorsal telencephalon does grow, albeit not nearly to normal size. We suggest two interpretations for the apparently different effects of the BF-1 mutation on dorsal and ventral telencephalon development. First, it is possible that the BF-1 expression pattern does not reflect its function. BF-1 may have different functions in the dorsal and ventral regions of telencephalon. It may be that measurements of BF-1 mRNA levels do not reflect the levels in BF-1 protein expression,

or that different forms of the protein are expressed. However, the three different BF-1 transcripts we find in brain all encode the same protein (H. Li and E. L., unpublished data). Different functions of BF-1 in dorsal and ventral telencephalon may be due to interactions with regionally restricted molecules that modulate its activity. For example, a ventral telencephalon–specific coactivator may enhance the activity of BF-1 in the ventral telencephalon, thus accounting for a more severe ventral phenotype in the BF-1 mutant.

We favor a second possible interpretation of the data, which postulates a common function for BF-1 throughout the telencephalon in promoting cell proliferation. The dorsal and ventral regions of the telencephalon normally differ in their proliferative potential as well as in the timing of neuronal differentiation. The dorsal telencephalic neuroepithelium has a delayed onset of neuronal differentiation compared with that of the ventral region, which leads to a longer period of neuroepithelial cell proliferation and greater growth of the dorsal telencephalon (Altman and Bayer, 1995). We believe that these features and the phenotype of the BF-1 deletion both suggest that BF-1 is not the sole regulator of cell proliferation in the developing telencephalon. In our model, BF-1 does not control the differential growth of the dorsal versus ventral telencephalon, but rather it functions to enhance the growth of the entire telencephalon. Although BF-1 is essential for neuroepithelial cells of the ventral telencephalon to proliferate, dorsal neuroepithelial cells must contain additional independent regulators that enable them to proliferate to a limited degree in the absence of BF-1. It is possible that regulators of proliferation are also restricted to subregions of the dorsal telencephalon, thus accounting for the subtle variations in cell proliferation found within this region. Dorsal-specific transcription factors such as Emx1 and Emx2 are candidates to be independent regulators of proliferation in the dorsal telencephalic neuroepithelium. Thus, we believe that normal regional differences in the growth of the telencephalon can be attributed to the combinatorial action of independently acting regulators of proliferation.

Cytokinetic studies of neuorepithelial cells have demonstrated a progressive increase in cell cycle length in the cerebral cortical region during the period of neuronal differentiation (E11–E17) in the mouse. This increase is due largely to an increase in the length of G1 (Hoshino et al., 1973). These changes are associated with an increase in the fraction of cells exiting the cell cycle and with depletion of the cerebral cortical neuroepithelium (Takahashi et al., 1992). The normal decline in the expression levels of BF-1 during the period of neurogenesis may control the progressive depletion of the cerebral cortical neuroepithelium in normal development. Thus, we postulate that BF-1 normally functions to enhance cell proliferation by facilitating cell cycle progression. Such a function for BF-1 is reminiscent of the activities of mitogens. We suggest that BF-1 may act by enhancing the response of neuroepithelial cells to mitogenic signals or, more directly, by regulating the activity of the components of the cell cycle machinery. These actions of BF-1 ultimately control the size of the telencephalon relative to the rest of the CNS. It is tempting to speculate that changes in the activity of BF-1 may be implicated in the encephalization of the brain during evolution.

Winged Helix Transcription Factors and Morphogenesis

While major advances have been made in identifying the genes specifying positional information in the early embryo, our understanding of how this information is translated into the control of morphogenesis remains limited. An essential component of morphogenesis is the precise regulation of the rate and duration of the proliferation of progenitor cells. Our studies of the BF-1 mutant strongly implicate this gene in the control of the proliferation and timing of differentiation of neuroepithelial precursor cells of the telencephalon. A large number of transcriptional regulators, including many WH genes, have been described with highly restricted patterns of expression during embyrogenesis. While transcription factors with restricted patterns of expression in adult tissues are usually associated with the regulation of tissue-specific genes, such factors may have different roles during development. Our results support a model in which morphogenesis is controlled through the combined actions of multiple transcriptional regulators with highly restricted expression patterns. We suggest that these factors regulate common targets that control cell division, thereby establishing temporal and spatial control of cell proliferation. A similar paradigm has been described from the studies of *string* function and regulation during Drosophila development. Cell proliferation in the early embryos is regulated by expression of *string*, which encodes a cdc25-type phosphatase that activates cdc2 (Edgar and O'Farrell, 1989; Millar and Russell, 1992). Recent studies show that many known patterning genes act locally to influence *string* expression and thereby control cell cycle progression (Edgar et al., 1994). Similar developmental control of the G1–S transition through the regulation of the expression of cyclin E has also been described (Duronio and O'Farrell, 1994; Knoblich et al., 1994). In mammals, *Hox* genes have been postulated to provide region-specific regulation of the local growth of precursor cells based on the morphogenetic consequences of targeted gene disruptions (Dolle et al., 1993; Condie and Capecchi, 1994). Thus, we speculate that BF-1 and other WH factors may translate positional information into the control of morphogenesis by independently regulating the expression of components of the cell cycle machinery in selected sets of progenitor cells. The activity of WH transcription factors could therefore serve to control the size of different structures and organs by controlling the number and timing of cell divisions in the precursor cells.

Experimental Procedures

Targeting of the BF-1 Locus and Generation of Homozygous BF-1 (−/−) Animals

Genomic 129/Ola clones containing the entire BF-1 gene were isolated from a λGEM library (provided by A. Berns). Restriction endonuclease

and partial sequence analysis were used to identify the BF-1 coding sequence. The targeting vector was constructed by fusing a *lacZ* cassette containing an intron to an NcoI site encoding amino acid 13 (Met) of the BF-1 coding sequence. This site had been previously generated by site-directed mutagenesis in a SalI–KpnI genomic fragment containing the BF-1 transcription initiation site. The PGK–*neo* cassette from the plasmid pKJ1 (M. McBurney; Tybulewicz et al., 1991), which contains the neomycin resistance gene (*neo*) under the control of the phosphoglycerate kinase (PGK) promoter was then ligated to the 3′ end of the *lacZ* cassette. The SmaI–NdeI fragment from the 3′ untranslated sequence of the BF-1 cDNA was ligated to the 3′ end of the PGK–*neo* cassette. The entire fragment with a 5′ SalI and 3′ XhoI site was then cloned at the SalI site into a pGEM4 plasmid containing the BamHI–SalI BF-1 genomic fragment. The 5′ BamHI site was previously changed to an SfiI site with linkers, to provide a unique site for linearization of the plasmid.

We used both CJ7 (Swiatek and Gridley, 1993) and J1 (Lee et al., 1992) ES cells, generously provided by Drs. T. Gridley and R. Jaenisch, respectively. Recombinant ES cells were generated essentially as described previously (Swiatek and Gridley, 1993). Briefly, 10^7 ES cells were electroporated with 30 μg of linearized targeting vector using a Bio-Rad Gene Pulser (250 μF; 250 V). Cells were then plated into 10 cm tissue culture dishes at a density of 5×10^5 cells per dish. G418 (Geneticin, GIBCO) selection was applied 24 hr after transfection at 250 μg/ml, and resistant colonies were isolated after 7–8 days. Disaggregated colonies were plated into wells of 96-well plates containing embryonic fibroblast feeder cells. Two-thirds of the cells were plated for PCR screening, and one-third were plated for future expansion. All the cells were grown in G418 selection media. Resistant clones were analyzed by PCR as described (LeMouellic et al., 1990) with the PGK primer 5′-AGTATTGTTTTGCCAAGTTCTAAT-3′ and the BF-1 primer 5′-TCCTATAAGTTGAATGGTATTTTG-3′. Approximate primer positions are indicated in Figure 1A. These primers yield a product of 1.1 kb with homologous recombinants. PCR was carried through 40 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. Positive clones by PCR were expanded for karyotype analysis, genomic Southern analysis, and injection into blastocysts. We obtained a total of 10 correctly targeted lines from 950 G418-resistant clones.

ES cell colonies positive for homologous recombination at the BF-1 locus were resuspended in injection medium (DMEM with 10 mM HEPES buffer and 10% fetal bovine serum). ES cells (10–15) were injected into the blastocoel cavity of 3.5 days postcoitum blastocysts isolated from C57BL/6J female mice. The injected blastocysts were then implanted back into the uteri of pseudopregnant B6CBAF1/J or CD1 (Jackson Laboratories) females at 2.5 days postcoitum. The resulting male chimeras with extensive ES cell contribution to the coat were bred with C57BL/6J females, and germline transmission was identified initially by the presence of agouti progeny. Mice heterozygous for the BF-1 mutation were intercrossed to generate homozygous BF-1 (−/−) animals.

Genotyping by Southern Analysis and PCR

Genomic DNA was obtained from ES cells, mouse tail tips, and embryonic sacs as previously described (Hogan et al., 1986; Bonnerot and Nicolas, 1993). Southern blot analysis was performed to confirm that the correct homologous recombination event, with the replacement of the BF-1 coding sequence, had occurred. Genomic DNA (7–8 μg) was digested with the restriction enzymes BamHI or KpnI. DNA was blotted onto Zeta-Probe GT membranes (Bio-Rad). Hybridization and washing was carried out according to the manufacturer's instructions. Membranes were hybridized to three probes: one 5′ to the targeted sequence (KpnI–HindIII BF-1 genomic fragment), one 3′ to the targeted sequence (SmaI–NdeI BF-1 genomic fragment), and one to the *lacZ* DNA (EcoRI–EcoRV fragment).

Routine genotyping of mice was performed by PCR. Primer sequences for BF-1 were BF1-U (5′-GGGCAACAACCACTCCTTCTCCAC-3′) and BF1-L (5′-GACCCCTGATTTTGATGTGTGAAA-3′). The expected size of the BF-1 product was 295 bp. We also used previously described primers to identify the *lacZ* gene (Bonnerot and Nicolas, 1993). PCR cycling conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 90 s, for a total of 30 cycles.

Histology and β-gal Staining

Embryos were obtained from timed pregnancies, with noon of the plug date defined as E0.5. Embryos were fixed in 4% paraformaldehyde. E16.5 and older embryos were perfusion-fixed by cardiac puncture. Paraffin embedding was performed by dehydrating embryos through ethanol and Histoclear (National Diagnostics) prior to immersion in paraplast (Fisher Scientific). Sections of 8 μm were stained with hematoxylin and eosin.

β-gal staining in 10 μm cryostat sections and whole embyros was performed as previously described (Bonnerot and Nicolas, 1993). Sections were stained for 2–8 hr and counterstained with Nuclear Fast Red. Whole embryos were stained for 30 min to 24 hr. Heterozygous specimens were stained for twice as long as homozygous specimens to compensate for the number of copies of β-gal. Selected stained whole embryos were subsequently embedded in paraffin for sectioning and counterstaining with Nuclear Fast Red.

In Situ Hybridization

Embryos were fixed in 4% paraformaldeyde and embedded in paraffin. Sections (8 μm) were processed for in situ hybridization with digoxigenin-labeled probes as previously described, except for the following changes (Schaeren-Wiemers and Gerfin-Moser, 1993). Digoxigenin-labeled cRNA probes (15 nM) were hybridized at 58°C. Anti-digoxigenin antibody (Boehringer Mannheim) diluted to 1:5000 was incubated with the tissue overnight. Probe templates were kindly provided by E. Boncinelli (Emx1 and Emx2; Simeone et al., 1993), P. Gruss (Pax6; Walther and Gruss, 1991), and J. Rubenstein (Dlx1 and Dlx2; Porteus et al., 1991).

BrdU Incorporation and Immunohistochemistry

For immunohistochemical detection of BrdU (Gratzner, 1982), BrdU (Sigma) was injected intraperitoneally into pregnant mice (30 mg/kg) 2 hr before sacrifice. Embryos were fixed in 4% paraformaldehyde for 4 hr and embedded in paraffin. Sections (8 μm) were treated with 5 μg/ml proteinase K at 37°C for 10 min, followed by denaturation of the DNA in 1 N HCl at 55°C for 10 min. After washing in PBS, sections were immersed in 0.1% H_2O_2 in PBS. Sections were then treated with avidin and biotin blocking solutions (Vector Laboratories) prior to blocking with 10% normal rabbit serum in PBS containing 0.5% Triton X-100 (PBS–TX). Sections were then incubated at 4°C overnight with rat monoclonal anti-BrdU antibody (clone B1/75; Sera Lab) diluted 1:50 in PBS–TX with 1% normal rabbit serum. The following day sections were incubated with biotinylated rabbit anti-rat antibodies diluted 1:200 in PBS with 1.5% normal rabbit serum for 30 min at room temperature, followed by incubation with avidin conjugated with peroxidase for 30 min at room temperature. Peroxidase reaction was developed with diaminobenzidine and H_2O_2 (Sigma) in PBS. Sections were counterstained lightly with hematoxylin.

For MAP2 immunohistochemistry, sections were blocked with 10% normal goat serum (Jackson ImmunoResearch Labs) in PBS–TX and then incubated at 4°C overnight with mouse monoclonal anti-MAP2 antibodies (clone HM-2; Sigma) diluted 1:250 in PBS–TX with 1% normal goat serum. Sections were then incubated with goat anti-mouse antibodies conjugated with peroxidase (Jackson ImmunoResearch Labs) diluted 1:50 in PBS–TX with 1% normal goat serum. Sections were then processed as above.

Acknowledgments

All correspondence should be addressed to E. L. We are grateful to the staff of the Transgenic Facility for help with mouse breeding. We thank T. Gridley and R. Jaenisch for the ES cell lines; A. Berns for the mouse 129 genomic library; E. Boncinelli, P. Gruss, and J. L. Rubenstein for probes; V. Hatini for help with gene targeting; K. Manova for advice on in situ hybridization; and E. Lacy and M. Jasin for helpful discussions. We also thank J. Massague, M. Ehrlich, C. A. Mason, and M. E. Hatten for critical comments on the manuscript. This work was supported by the National Institutes of Health RO1 HD29584 and an American Cancer Society Junior Faculty Research Award to E. L., a National Research Service Award to C. A. B., and a National Cancer Institute Cancer Center Support Grant to the Memo-

rial Sloan-Kettering Cancer Center.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received December 7, 1994; revised April 1, 1995.

References

Altman, J., and Bayer, S. (1995). Atlas of Prenatal Rat Brain Development (Boca Raton, Florida: CRC Press).

Angevine, J., and Sidman, R. (1961). Autoradiographic study of cell migration during histogenesis of the cerebral cortex in the mouse. Nature *192*, 766–768.

Berry, M., Rogers, A., and Eayres, J. (1964). Pattern of cell migration during cortical histogenesis. Nature *205*, 591–593.

Bonnerot, C., and Nicolas, J. (1993). Application of LacZ gene fusions to postimplantation development. In Guide to Techniques in Mouse Development (San Diego, California: Academic Press), pp. 451–469.

Caviness, V., and Sidman, R. (1973). Time of origin of corresponding cell classes in the cerebral cortex of normal and *reeler* mutant mice. J. Comp. Neurol. *148*, 141–152.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P., and Gruss, P. (1993). Pax: gene regulators in the developing nervous system. J. Neurobiol. *24*, 1367–1384.

Clark, K., Halay, E., Lai, E., and Burley, S. (1993). Cocrystal structure of the HNF-3/*fork head* DNA-recognition motif resembles histone H5. Nature *364*, 412–420.

Condie, B., and Capecchi, M. (1994). Mice with targeted disruptions in the paralogous genes *hoxa-3* and *hoxd-3* reveal synergistic interactions. Nature *370*, 304–307.

Crandall, J., Jacobson, M., and Kosik, K. (1986). Ontogenesis of microtubule associated protein 2 (MAP-2) in embryonic mouse cortex. Dev. Brain Res. *28*, 127–133.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., and Duboule, D. (1993). Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. Cell *75*, 431–441.

Duronio, R., and O'Farrell, P. (1994). Developmental control of a G1-S transcriptional control program. Development *120*, 1503–1515.

Edgar, B., and O'Farrell, P. (1989). Genetic control of cell division patterns in the Drosophila embryo. Cell *57*, 177–187.

Edgar, B., Lehman, D., and O'Farrell, P. (1994). Transcriptional regulation of *string* (*cdc25*): a link between developmental programming and the cell cycle. Development *120*, 3131–3143.

Figdor, M. C., and Stern, C. D. (1993). Segmental organization of the embryonic diencephalon. Nature *363*, 630–634.

Gratzner, H. (1982). Monoclonal antibodies against 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science *218*, 474–475.

Hatini, V., Tao, W., and Lai, E. (1994). Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. J. Neurobiol. *25*, 1293–1309.

Hofman, M. (1989). On the evolution and geometry of the brain in mammals. Prog. Neurobiol. *32*, 137–158.

Hogan, B., Lacy, E., and Constantini, F. (1986). Manipulating the Mouse Embryo (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Hoshino, K., Matsuzawa, T., and Murakami, U. (1973). Characteristics of the cell cycle of matrix cells in the mouse embryo during histogenesis of the telencephalon. Exp. Cell Res. *77*, 89–94.

Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A., and Rossant, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the En-2 homeobox. Science *251*, 1239–1243.

Keynes, R., and Lumsden, A. (1990). Segmentation and the origin of regional diversity in the vertebrate central nervous system. Neuron *4*, 1–9.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R., and Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell *77*, 107–120.

Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M., and Lumsden, A. (1993). Hox homeobox genes and regionalisation of the nervous system. J. Neurobiol. *24*, 1328–1340.

Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H., and Darnell, J. E., Jr. (1990). HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. *4*, 1427–1436.

Lai, E., Prezioso, V. R., Tao, W., Chen, W. S., and Darnell, J. E., Jr. (1991). Hepatocyte nuclear factor 3α belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head*. Genes Dev. *5*, 416–427.

Lai, E., Clark, K., Burley, S., and Darnell, J. (1993). Hepatocyte nuclear factor-3/*fork head* or "winged helix" proteins: a family of transcription factors of diverse biologic function. Proc. Natl. Acad. Sci. USA *90*, 10421–10423.

Lee, K.-F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V., and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell *69*, 737–749.

LeMouellic, H., Condamine, H., and Brulet, P. (1990). Targeted replacement of the homeobox gene Hox 3.1 by the *E. coli* lacZ in mouse chimeric embryos. Proc. Natl. Acad. Sci. *87*, 4712–4716.

Li, J., and Vogt, P. (1993). The retroviral oncogene *qin* belongs to the transcription factor family that includes the homeotic gene *fork head*. Proc. Natl. Acad. Sci. USA *90*, 4490–4494.

Marin-Padilla, M. (1990). Three-dimensional structural organization of layer I of the human cerebral cortex: a Golgi study. J. Comp. Neurol. *299*, 89–105.

McMahon, A. P., Joyner, A. L., Bradley, A., and McMahon, J. A. (1992). The midbrain–hindbrain phenotype of *Wnt-1*$^{-}$/*Wnt-1*$^{-}$ mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. Cell *69*, 581–595.

Millar, J. B. A., and Russell, P. (1992). The cdc25 M-phase inducer: an unconventional phosphatase. Cell *68*, 407–410.

Porteus, M., Bulfone, A., Ciaranello, R. D., and Rubenstein, J. L. R. (1991). Isolation and characterization of a novel cDNA clone encoding a homeodomain that is developmentally regulated in the ventral forebrain. Neuron *7*, 221–229.

Price, M., Lemaistre, M., Pischetola, M., DiLauro, R., and Duboule, D. (1991). A mouse gene related to *Distal-less* shows a restricted expression in the developing forebrain. Nature *351*, 748–751.

Puelles, L., and Rubenstein, J. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. Trends Neurosci. *16*, 472–479.

Schaeren-Wiemers, N., and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: *in situ* hybridization using digoxigenin-labelled cRNA probes. Histochemistry *100*, 431–440.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A., and Boncinelli, E. (1992). Nested expression domains of four homeobox genes in developing rostral brain. Nature *358*, 687–690.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M., Nigro, V., and Boncinelli, E. (1993). Two vertebrate homeobox genes related to the *Drosophila empty spiracles* gene are expressed in the embryonic cerebral cortex. EMBO J. *12*, 2735–2747.

Swiatek, P. J., and Gridley, T. (1993). Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene *Krox20*. Genes Dev. *7*, 2071–2084.

Takahashi, T., Nowakowski, R., and Caviness, V. J. (1992). BUdR as an S-phase marker for quantitative studies of cytokinetic behavior in

the murine cerebral ventricular zone. J. Neurocytol. *21*, 185–197.

Tao, W., and Lai, E. (1992). Telencephalon-restricted expression of BF-1, a new member of the HNF-3/*fork head* gene family, in the developing rat brain. Neuron *8*, 957–966.

Thomas, K., and Capecchi, M. (1990). Targeted disruption of the murine *int-1* protooncogene resulting in severe abnormalitites in midbrain and cerebellar development. Nature *346*, 847–850.

Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-*abl* proto-oncogene. Cell *65*, 1153–1163.

Walther, C., and Gruss, P. (1991). *Pax-6*, a murine paired box gene, is expressed in the developing CNS. Development *113*, 1435–1449.