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The *flat-top* gene is required for the expansion and regionalization of the telencephalic primordium

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SUMMARY

The telencephalic vesicles form in the mouse embryo by the expansion of precursor regions in the anterior neural tube. Once the vesicles have formed, discrete dorsal and ventral territories can be recognized that later give rise to cortical and subcortical structures, respectively. To investigate the mechanisms that regulate the expansion and regionalization of the telencephalon, we have carried out a screen to identify recessive mutations that disrupt these events. We isolated a mouse mutant in which an early and critical step in development of the telencephalic vesicles is disrupted. Telencephalic primordia are present in flat-top embryos but they fail to progress to form the telencephalic vesicles. An increased rate of proliferation in the forebrain neurectoderm that accompanies telencephalic expansion in wild-type embryos fails to occur in flat-top embryos. Regionalization events that would normally take place during expansion of the primordia also fail to occur. Thus the phenotype of the flat-top mouse reveals that outgrowth of the telencephalic vesicles and their regionalization are coupled processes.

Key words: *flat-top*, Telencephalic vesicle, Mouse, Proliferation, Forebrain

INTRODUCTION

Substantial progress has been made in recent years in determining types and sources of inductive signals that regionalize the vertebrate neural tube. Despite these advances, the mechanisms that pattern the forebrain are still poorly understood and critical steps have not been clearly defined for many aspects of forebrain development. This is due in part to the complexity of the structures formed by the anterior neural tube. A case in point is the formation of the telencephalon, the precursor to the cerebral cortices, hippocampus, olfactory bulbs and basal ganglia. Patterning mechanisms acting on the neural plate produce telencephalic primordia, regions of the anterior neurectoderm that are fated to form the telencephalic vesicles (Couley and Le Douarin, 1988). The primordia rapidly expand to form vesicles which are in turn regionalized (Shimamura et al., 1995, 1997). The events that establish the telencephalic primordia, the regulatory mechanisms that govern their expansion and the patterning mechanisms that act on the vesicles are not understood.

Fate mapping studies in the chick indicate that the telencephalic primordia are small groups of cells at the anterior margin of the neural plate (Couley and Le Douarin, 1988). In mouse embryos, the telencephalic precursor region can be detected by its expression of the winged-helix transcription factor, BF-1 (Hatini et al., 1994; Shimamura and Rubenstein, 1997) and this early expression has provided the means to tease out some aspects of the earliest stages in telencephalic development. BF-1 expression in the neural plate is induced by FGF8 (Shimamura and Rubenstein, 1997), a secreted signaling molecule that is expressed at the anterior neural ridge (ANR), the anterior junction between the ectoderm and the neurectoderm (Crossley and Martin, 1995). The telencephalic vesicles themselves continue to express BF-1 as they expand rapidly immediately following neural tube closure. Expression of BF-1 in the brain is restricted to the telencephalon and the anterior half of the optic vesicle, consistent with the idea that its early expression is a useful marker for the telencephalic primordia (Hatini et al., 1994). Despite its usefulness as a marker of the telencephalic precursor region, BF-1 expression is not necessary for the formation of the vesicles. The telencephalic vesicles form in mice homozygous for a deletion of the *BF-1* gene but fail to develop after their initial formation (Xuan et al., 1995).

Regionalization of the telencephalon determines the structures that are formed from the primitive vesicles. The dorsal telencephalon gives rise to cortical structures such as the hippocampus, the cerebral cortices and the olfactory bulbs, while the ventral telencephalon gives rise to the basal ganglia and other subcortical structures. Regionally restricted gene expression can be detected as early as 9.5 dpc in the mouse and recent studies have provided information about the role of regionally restricted genes in cell fate choices during telencephalic development.

Pax6 and *Emx2* expression in the telencephalon is dorsally restricted (Boncinelli et al., 1993; Simeone et al., 1992; Walther and Gruss, 1991). *Pax6* plays a key role in the formation of boundaries between the dorsal telencephalon and

surrounding tissues (Stoykova et al., 1997). *Emx2*, in contrast, is necessary for determining the fate of the hippocampus and other limbic structures (Yoshida et al., 1997). Ventrally restricted genes include the *Nkx2.1*, *Shh* and *Dlx* genes (Bulfone et al., 1993; Dolle et al., 1992; Lazzaro et al., 1991; Price, 1993; Robinson et al., 1991; Shimamura et al., 1995). The role of *Shh* in the development of the telencephalon has not been determined. Knockout of the *Nkx2.1* gene reveals that its expression is required for proper development of the septal region and the basal ganglia (Kimura et al., 1996). Similar studies have shown that expression of either *Dlx1* or *Dlx2* is necessary for differentiation of striatal neurons (Anderson et al., 1997). BF-1 is also involved in ventral fate selection within the telencephalon. BF-1 is expressed in a ventral-to-dorsal gradient within the telencephalon and the BF-1$^{-/-}$ telencephalon fails to express *Dlx* genes in the ventral telencephalon (Xuan et al., 1995).

The application of gene targeting approaches to study genes such as *Emx2* or the *Dlx* genes, which have selective expression patterns in the telencephalic vesicles, has begun to provide information about regional fate choices during development of the telencephalon. This approach has not yet yielded information about how formation of the vesicles is regulated or about how regionalization of the vesicles occurs.

To elucidate earlier events in the regulation of telencephalic development, we have carried out a direct phenotypic screen for mutations that disrupt the development of the telencephalic vesicles. Random chemical mutagenesis coupled with a morphological screen for embryonic forebrain defects has allowed us to isolate four mutants with defective telencephalic development.

Here we describe the phenotype of one of the mutants, flat-top. Characterization of flat-top embryos reveals three defects in the development of the telencephalon; failure of telencephalic primordia to expand to form the vesicles, failure to regionalize the telencephalon and a failure to maintain the expression of Shh and BF-1 in the forebrain. The disruption of both expansion and regionalization of the telencephalon in flat-top embryos and the close temporal correlation of these events in wild-type embryos supports the hypothesis that regionalization and expansion are mechanistically coupled.

MATERIALS AND METHODS

Mutant screen

8- to 12-week-old male BTBR mice (JAX) were injected with a single dose of 250 mg/kg of ethyl-nitroso-urea (Sigma, MO) according to a protocol provided by Bill Dove. 10 weeks after injection, the mice were mated to female C57Bl/6J (JAX). Fertility in the treated males was typically recovered starting about 12 weeks after the injection. The G_1 male offspring resulting from this cross were mated to C57Bl/6J females. The G_2 females that resulted were superovulated at 6-8 weeks of age with intraperitoneal injections of 5-10 IU PMS-G (Sigma) followed by 5-10 IU hCG 46-48 hours later. The superovulated females were mated to their fathers overnight and checked for vaginal plugs the next morning. Embryos dissected from the uterus on the 10th day following observation of the plug were examined for forebrain defects under a stereomicroscope. In those cases where two or more embryos with characteristic forebrain defects were observed within a litter, the line was expanded by further mating of the G_1 male to additional C57Bl/6J females. G_2 females that resulted were again mated to their fathers in timed matings. G_2 males were crossed to C57Bl/6J females and the resulting G_3 females were mated to their G_2 fathers.

In situ hybridization

Digoxigenin- or fluorescein-labeled RNA probes were prepared according to the instructions of the manufacturer (Boehringer Mannheim). Whole-mount in situ hybridization was performed as described (Henrique et al., 1995), except that an RNase digestion step was inserted: immediately after overnight hybridization embryos were treated with 10 μg/ml RNase A in hybridization buffer for 30 minutes at 37°C.

The substrate used for single color reactions was Magenta-phos (Biosynth, Switzerland), used at 340 μg/ml. For double-label in situs, INT/BCIP (Boehringer Mannheim) was used as the second substrate. Embryos were documented using a Leica Wild-M420 macroscope a Sony DXC 96OMD CCD camera and a Data Translation Digitizer in a Macintosh Computer. Plasmids for in situ probes were kindly provided by Drs E. Lai (BF-1), G. Martin (*Fgf-8*), A. McMahon (*Shh*), P. Gruss (*Pax6* and *Six3*), A. Joyner (*En-2*) and J. Rubenstein (*Nkx2.1*, *Otx2* and *Emx2*).

Immunohistochemistry

Embryos were fixed at 4°C overnight in 4% paraformaldehyde. For sections, embryos were embedded in paraffin and cut at 10 μm. Endogenous peroxidase activity was blocked by a 30 minute incubation in 5% hydrogen peroxide. Metaphase cells were detected with a rabbit polycolonal antiserum directed against phosphorylated (Ser10) histone H3 (Upstate Biotechnology, Lake Placid NY). Labeled cells were counted for forebrain and hindbrain neurectoderm on serial sections through wild-type and mutant embryos at 9.5 dpc. Proliferation was assessed for the hindbrain and forebrain regions on the same set of sections. Neurectodermal length was measured using the NIH Image program.

Neuronal differentiation was detected with the anti-class III β tubulin monoclonal TuJ1 (Promega). Both antibodies were used in combination with the Vectastain Elite ABC kits (Vector Laboratories) as directed by the manufacturer. A TUNEL labeling kit, Apoptag (Oncor) was used to reveal cells undergoing programed cell death.

Mapping

A set of 96 simple sequence length polymorphism (SSLP) markers (Dietrich et al., 1996) spaced at 20 cM intervals throughout the genome was used for initial linkage evaluation. The full set of 96 markers was used to score eight DNAs from flat-top heterozygous carriers (G_2 males and females and G_3 females). Based on this experiment, some markers were excluded from further analysis and a reduced set of markers was scored on an additional 8 DNAs: 5 from carriers and 3 from non-carriers. This analysis allowed us to identify two linked markers on chromosome 4. Additional polymorphic markers on chromosome 4 were subsequently tested together with additional DNAs from heterozygous carriers. Chromosome 4 markers were also mapped using a set of 94 radiation hybrid DNAs purchased from Research Genetics. Both meiotic and radiation hybrid data were analyzed using the MapManager QTb20ppc program (http://mcbio.med.buffalo.edu/mapmgr.html).

RESULTS

Isolation of flat-top

To isolate novel mouse mutants with defects in the development of the forebrain we used random chemical mutagenesis and a morphological screen (Fig. 1). We first treated male BTBR mice with ethyl-nitroso-urea (ENU), an alkylating agent and high efficiency mutagen. Treatment with

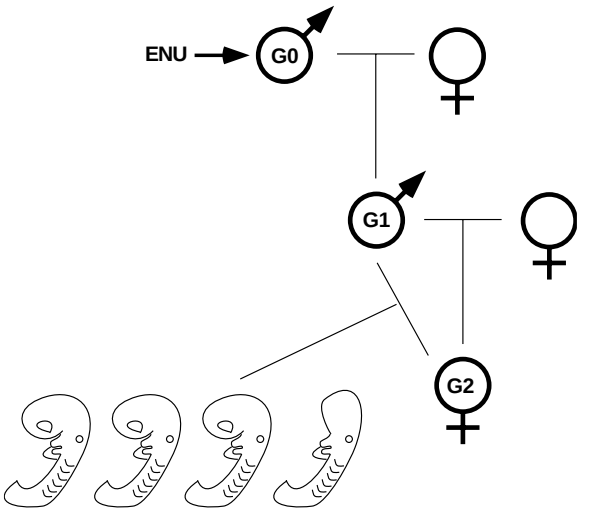

Fig. 1. A screen for recessive embryological mutations. Male BTBR mice (G_0) were treated with ethyl-nitroso-urea. 12 weeks later they were crossed to C57Bl/6J females. The G_1 males that resulted were then crossed to C57Bl/6J females and G_2 females were saved. At 7 weeks of age, the G_2 animals were backcrossed to their fathers to uncover recessive mutations. Embryos were dissected out from the uterus and examined for defects at 10.5 days of gestation. 150 lines, comprising G_1 males and their G_2 female offspring, were screened during the isolation of the *flat-top* mutant.

ENU causes the mice to enter a sterile period after which they produce gametes carrying induced mutations. Treated mice (G_0) were crossed to C57Bl/6J (B6) females and approximately 150 male offspring (G_1) were produced. At 7 weeks of age each G_1 male was mated to a B6 female to produce female (G_2) offspring. As each G_2 female reached 7 weeks of age, it was superovulated and mated to its father. The embryos from these timed pregnancies were dissected from the uterus and examined for possible defects at 10.5 days postcoitum (dpc). From these efforts, we identified four mutations that disrupted the development of the forebrain. We characterized one of these mutations, which defines the flat-top locus, in more detail because it appeared to block the development of the telencephalon at an early stage.

Localization of the *flat-top* gene

To provide the basis for efficient breeding of *flat-top* carriers and to evaluate possible candidate genes, we determined the chromosomal location of the *flat-top* gene. Crosses between *flat-top* carriers and C57Bl/6J were carried out. The progeny from the crosses were either back-crossed or inter-crossed and embryos were examined at 9.5-10.5 dpc to determine whether mutant embryos were present. Animals that were clearly heterozygous carriers were then scored for a set of 96 simple sequence length polymorphism (SSLP) markers spaced at 20 cM intervals. Analysis of an initial set of eight animals provided clear evidence of linkage to the distal portion of chromosome 4 in a region of synteny with human chromosome 1p35-36. Scoring of additional markers and ~1500 additional meioses allowed the gene to be localized to an interval of 0.3±0.15 cM (Fig. 2). Polymorphic markers in this region were ordered both on the panel of *flat-top* meiotic recombinants and on a panel of radiation hybrid (RH) DNAs. Several mouse genes whose human homologues are on 1p35-36 were also mapped on the RH panel to allow alignment of the *flat-top* meiotic map with human transcript maps (Jensen et al., 1997) and with the emerging human genomic sequence of this area of the human genome (http://www.sanger.ac.uk/HGP/Chr1/). Examination of mouse and human genetic maps does not reveal any obvious candidates for the *flat-top* gene.

The flat-top phenotype

Flat-top embryos are recognizeable after 8.5 days of gestation because of characteristic defects that result from failure of the embryos to turn or rotate around the body axis. Mutant embryos are smaller than their littermates, in part because of a developmental delay of 2-5 somites. Even when matched by somite number, however, they are 60-70% of wild-type size. The embryos otherwise appear to develop normally until about 9-9.5 dpc when the failure of the telencephalic vesicles to form becomes apparent. Despite the obvious defect in telencephalic development, the forebrain does increase in size in flat-top embryos during this time period (compare Fig. 3B and D). Neuromeric constrictions marking the rhombomeres of the hindbrain and the isthmic constriction appear normally. Constrictions between the midbrain and the caudal diencephalon and between the caudal and rostral diencephalon appear more prominently than normal and, by about 10.5 dpc, buckling of the neuroepithelium in the forebrain is common. Other defects include a kinked neural tube in the spinal cord that may be secondary to the failure to turn. Flat-top embryos begin to die by about 11.5 dpc apparently because of problems that result from the failure to turn.

Failure of telencephalic evagination

The telencephalic vesicles are formed by the expansion of small precursor regions dorsal and anterior to the optic vesicles. Following neural tube closure, the telencephalic

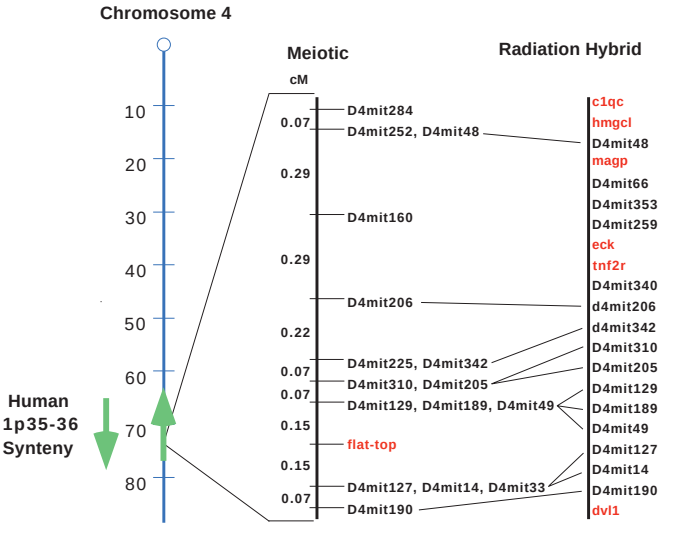

Fig. 2. Mapping of the *flat-top* locus to chromosome 4. The *flat-top* mutation has been mapped meiotically to the distal portion of chromosome 4 in a region of synteny with human chromosome 1p35-36. Radiation hybrid mapping was used to determine the most likely order of MIT markers and of mouse orthologues of genes (shown in red) that are on the human transcript map.

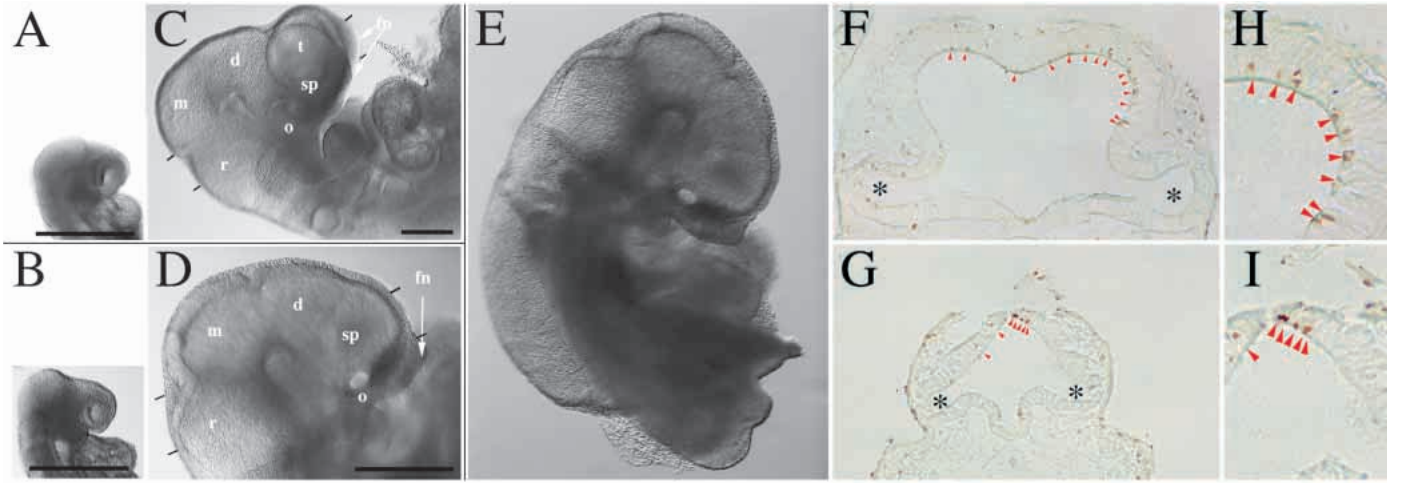

Fig. 3. The flat-top phenotype. Wild-type (A) and flat-top (B) embryos at 9-10 somites. Embryos that are 24 hours older are shown in C and D. The telencephalic vesicles fail to form in flat-top embryos. Constrictions of the neural tube that demarcate the major subdivisions of the anterior central nervous system are apparent in both wild-type and flat-top embryos. The failure of flat-top embryos to turn is apparent in E. Tick-marks in B and D indicate the regions that were sectioned for immunohistochemical labeling of metaphase cells (summarized in Table 1). Representative sections are shown in F-I. Red arrowheads indicate labeled cells. The optic vesicles are indicated by asterisks. Abbreviations: r, rhombencephalon; m, mesencephalon; d, diencephalon; t, telencephalon; sp, secondary prosencephalon; o, optic vescle; fn, frontonasal mass.

precursor regions grow more rapidly than the surrounding neurectoderm, such that between ~8.75 and 10.0 days discrete vesicles are formed. To determine whether defects in cell proliferation might be responsible for the failure of the telencephalic vesicles to form, we labeled metaphase cells with an antisera against phosphorylated histone H3 (Hendzel et al., 1997). Quantification of metaphase cells in the forebrain is summarized in Table 1. For comparsion, proliferation in the hindbrain was also assessed on the same set of sections. Wild-type embryos had a mitotic index in the forebrain region that was 1.5 times that in the hindbrain region (P<0.1), consistent with the fact that the telencephalon is rapidly expanding relative to the neural tube as a whole during this time period. In contrast, the rate of proliferation in the forebrain of flat-top embryos was lower and essentially the same as in the hindbrain, suggesting that the failure of telencephalic evagination is the result of an inability to upregulate proliferation in the telencephalon.

We also investigated the possibility that increased rates of differentiation or apoptosis could play a role in the flat-top phenotype. Differentiation was assessed with a monoclonal antibody directed against class III β-tubulin (Easter et al., 1993; Moody et al., 1989) and apoptotic cells were detected using the TUNEL labeling technique (Gorczyca et al., 1993). Neither of these assays uncovered a difference between mutant and wild-type embryos. Neuronal differentiation was not detectable in the telencephalon of either wild-type or flat-top embryos during the period of vesicle formation (data not shown). Similarly, neither the pattern nor the rate of apoptosis was affected by the mutation (data not shown).

Anterior identity is normally specified

Proliferation in the early mammalian embryo is dependent upon patterning signals. To determine whether the apparent proliferative deficit in the telencephalon of flat-top embryos was indicative of more general patterning defects in the anterior portion of the CNS, we examined the expression of a number of genes at 9-10 dpc. For this analysis, we chose genes whose expression is restricted to the anterior portion of the central nervous system at an early stage. The expression of two transcription factors *Otx2* and *Six3* was examined first (Fig. 4A-F). *Otx2* expression is found throughout the presumptive forebrain and midbrain regions of the neural plate and persists in these areas at 9.0 dpc (Acampora et al., 1995; Ang et al., 1996; Rhinn et al., 1998; Simeone et al., 1992). *Six3* expression begins at the anterior margin of the neural plate where it encompasses or overlaps the telencephalic precursor region (Oliver et al., 1995). At 9.0 dpc, the highest levels of *Six3* expression are found at the anterior midline and in the optic vesicles. The expression pattern of both of these genes is normal in flat-top embryos indicating that the flat-top defects are not due to a general failure to establish anterior identity.

We next examined the expression of the *BF-1* gene as a marker of the telencephalic vesicles. At 9.0-10.5 dpc in wild-type embryos, BF-1 is expressed in a gradient, with high levels of expression in the ventral telencephalon that steadily decrease towards the dorsal telencephalon (Fig. 4H,J,L). The expression of BF-1 in flat-top embryos is found at high levels in the ventral telencephalic region but does not extend into the dorsal telencephalon as it does in the wild-type (yellow arrowheads in Fig. 4I,J). By 10.5 dpc, expression of BF-1 has almost completely disappeared from the neuropeithelium of

Table 1. Cell proliferaion in wild-type and flat-top neuroectoderm

Wild type		Flat-top	
Forebrain	Hindbrain	Forebrain	Hindbrain
14.9±1.7	8.8±0.6	8.0±1.6	8.3±0.8

Values are number of labeled cells/100 μm±standard error. A total of 1173 cells were counted in 19 stained sections from 3 wild type embryos. 264 cells in 16 sections were counted from 3 flat-top embryos. The regions of the embryos that were assessed for proliferation is indicated in Fig. 3C and D.

mutant embryos (Fig. 4K). Thus the failure of the telencephalic vesicles to evaginate is correlated with the loss of expression of a marker of the telencephalon and its precursor region.

The role of Fgf8

It was recently demonstrated that FGF8, a secreted signaling molecule, is capable of inducing the expression of BF-1 in the anterior portion of the neural plate (Shimamura and Rubenstein, 1997). FGF8 is also necessary for the expression of the *engrailed* gene homologues *En1* and *En2* in more posterior regions that correspond to the presumptive midbrain (Crossley et al., 1996; Reifers et al., 1998). A defect in FGF8 signaling might plausibly underlie the failure of telencephalic development seen in flat-top embryos. A general defect in FGF8 signaling would be expected to lead to the loss of *En* gene expression in the midbrain as well as BF-1 expression in the forebrain. To examine this possibility we looked at the expression of *En2* in the midbrain at 10.5 dpc (Fig. 5). Both wild-type and flat-top embryos show similarly robust gradients of expression emanating from the isthmic constriction (Fig. 5). Thus any defect in FGF8 signaling in flat-top embryos is relatively specific to the forebrain. A logical mechanism would be the loss of *Fgf8* expression in the anterior neurectoderm while expression at the isthmic constriction is maintained.

Expression of *Fgf8* in ~5 somite embryos has been described at the anterior margin of the neural plate, in the anterior neural ridge (ANR). At later stages, *Fgf8* is expressed in the commissural plate (CP) at the anterior midline of the telencephalon. Since BF-1 expression is initiated at early stages but is subsequently lost it seemed possible that a defective transition from ANR to CP expression was responsible for the loss of BF-1 expression in flat-top embryos. Careful examination, however, indicated that the transition from ANR to CP happens well before the time at which BF-1 expression is lost in flat-top embryos (data not shown and Scott May, personal communication, summarized in Fig. 8). Thus the transition from ANR to CP plate expression is not temporally correlated with the loss of BF-1 expression. Based on this we examined the expression of *Fgf8* in flat-top embryos at ~9.5 and 10.5 dpc (Fig. 5C-F). Normal expression of *Fgf8* at the CP was found at both times indicating that a loss of *Fgf8* expression was not responsible for the loss of BF-1 expression and for the failure of telencephalic evagination.

Regionalization of the expanding telencephalon

Regionally restricted gene expression within the telencephalic vesicles is critical for their proper development. The failure of flat-top embryos to express BF-1 in the dorsal telencephalon may be indicative of more general defects in the regionalization of the telencephalon. To examine this, we first defined the expression patterns of a set of dorsal and ventral markers in wild-type embryos, before during and after the formation of the telencephalic vesicles. *Emx2* and *Pax6* were examined as genes whose expression is dorsally restricted and *Nkx2.1* and *Shh* as ventrally restricted genes. Expression of all four genes in the telencephalic primordia, prior to vesicle formation, is shown in Fig. 6.

Expression of *Emx2* is restricted to the presumptive dorsal telencephalon from about the 3-somite stage onwards. Expression is initially seen in the region of the neural plate that is fated to form the dorsal telencephalon. After telencephalic evagination *Emx2* expression is clearly restricted to the dorsal telencephalon. Expression in the wild-type dorsal telencephalic primorda is shown in Fig. 6A.

Pax6, on the contrary, is expressed at only low levels in the telencephalic primordia (Fig. 6B). Early and low level expression throughout the anterior and lateral portions of the 3- to 5-somite neural plate quickly changes as the posterior portions of the expression are upregulated. The relative increase in expression is correlated with the appearance of the

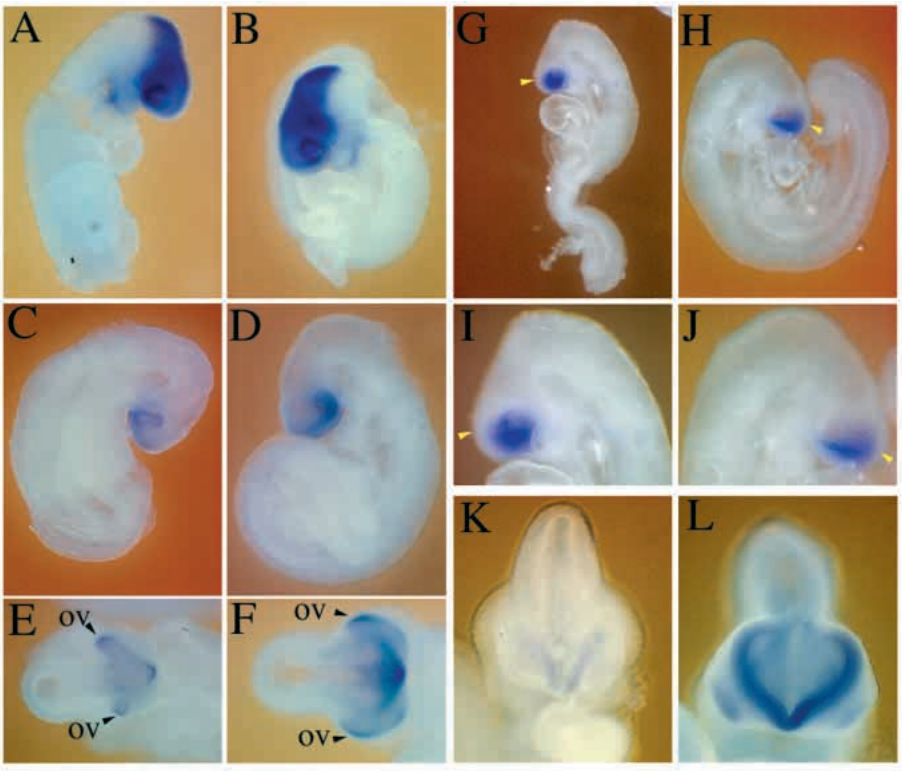

Fig. 4. The *flat-top* gene is not required for anterior forebrain identity. Whole-mount in situ hybridization of flat-top (A,C,E,G,I,K) and wild-type (B,D,F,H,J,L) embryos with forebrain markers. The spatial pattern of expression of the anteriorly restricted genes *Otx2* (A,B; *n*=3) and *Six3* (C-F; *n*=3) is normal in flat-top embryos although the level of *Six3* expression is lower in the mutant. This can be seen in both the side views (A-D) and in the dorsal views of the heads (E,F). The expression of BF-1 in contrast is drastically affected by the mutation (G-L). At 9.0 dpc, BF-1 is expressed at high levels in the ventral telencephalon but expression is absent from the dorsal telencephalon (yellow arrowheads in G,I; *n*=4). In contrast, expression in wild-type embryos is present in both the ventral and the dorsal telencephalon (yellow arrowheads in H,J). Expression of BF-1 is nearly extinguished by 10.5 dpc in flat-top embryos (front view of head in K; *n*=5). In wild-type embryos robust expression is present (front view of head in L).

optic evaginations and *Pax6* expression is found in the presumptive dorsal diencephalon and in the lateral portions of the optic evagination. As the telencephalic vesicles expand, the expression of *Pax6* in the dorsal telencephalon is upregulated such that by about 10.0 dpc similar levels of expression are seen in the diencephalon and in the telencephalon. Thus high level expression of *Pax6* in the dorsal telencephalon is correlated with telencephalic expansion and is a late event relative to the establishment of *Emx2* expression.

The ventral markers that we examined, *Nkx2.1* and *Shh*, have their onset of expression in the ventral telencephalon only after the telencephalon has begun its expansion. *Nkx2.1* is expressed in two domains in the forebrain. An early domain in the ventral diencephalon (Fig. 6D) is augmented, as the expansion of the primordia to form the vesicles begins, by expression in the ventral telencephalon, in an area that is thought to form the pallidum and septal region (Shimamura et al., 1995).

Shh is expressed in the ventral part of the neural tube throughout the neuraxis. Expression in the anterior forebrain neurectoderm is marked at its posterior side by a prominent dorsal deflection at the zona limitans, the transition to the prechordal region. Telencephalic expression begins shortly after the expansion of the primordia to form the vesicles, apparently by spreading from the diencephalic expression domain.

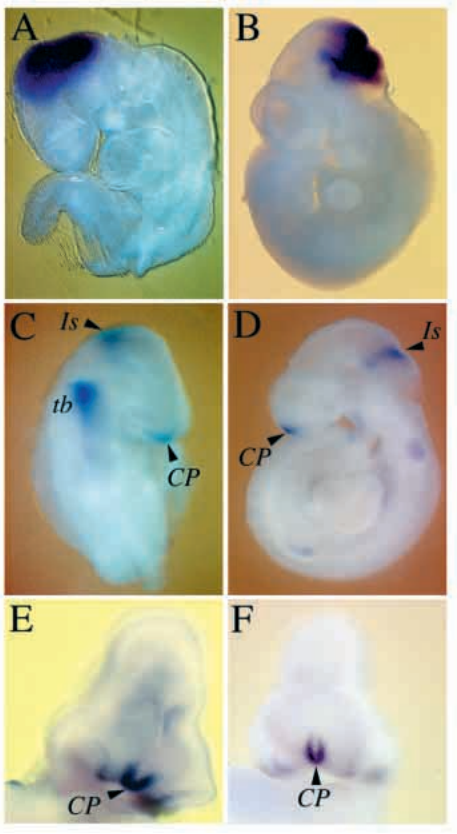

Fig. 5. The failure of telencephalic expansion is not due to a loss of *Fgf8* expression or the FGF8 responsiveness of the neurectoderm as a whole. Whole-mount in situ hybridization of flat-top (A,C,E) and wild-type embryos (B,D,F) with *En2* or *Fgf8* probes. FGF8 signalling in the neurectoderm is not generally compromised as evidenced by the normal expression of *En2* in flat-top embryos (compare A and B; *n*=2). *Fgf8* is expressed normally at the commissural plate during the time period over which BF-1 expression decays. Side views of Fgf8 expression in a flat-top embryo and in a wild-type littermate at 9.5 dpc are shown in C and D (*n*=2). Front views of the commissural plate expression at 10.5 dpc are shown in E and F (*n*=2). Abbreviations: Is, Isthmus; tb, tail-bud; CP, commissural plate.

Regionalization is defective in flat-top embryos

The expression patterns of all four genes in flat-top embryos at 9.5 dpc was similar to that in wild-type embryos at an earlier (~8.5 dpc) stage of development. So for example, *Emx2* expression in flat-top embryos is restricted to the dorsal region of the anterior neural tube (Fig. 7C and G). *Pax 6*, on the contrary, was expressed at higher levels in the dorsal diencephalon than in the dorsal telencephalon. This is similar to the wild-type situation at ~8.5-8.75 dpc but, in contrast to the normal pattern at 9.5 dpc where expression is at similar levels in both the diencephalon and in the telencephalon. The expression pattern of both Nkx2.1 and SHH in flat-top embryos at 9.5 dpc is again similar to that in younger wild-type embryos in that diencephalic expression is present but telencephalic expression is not.

To determine whether the apparent failure in telencephalic regionalization at 9.5 dpc was merely the result of a delay, we also examined the expression of these markers at 10.5 dpc. This analysis was consistent with the results with 9.5 dpc embryos with the exception of SHH. In a pattern that was surprisingly similar to the case of BF-1 expression, we found that flat-top embryos were unable to maintain the expression of *Shh* in the forebrain. At 9.0 dpc *Shh* expression was present along the extent of the neuraxis. By 10.5 dpc the domain of *Shh* expression

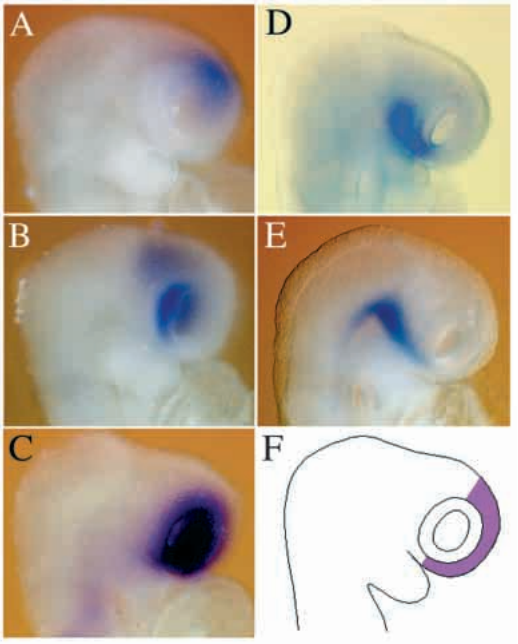

Fig. 6. *Emx2* expression is regionally restricted in the wild-type telencephalic primordia but expression of *Pax6*, *Nkx2.1* or *Shh* is not. Whole-mount in situ hybridization of 10- to 12-somite-stage wild-type embryos was carried out with probes for *Emx2* (A), *Pax6* (B), BF-1 (C), *Nkx2.1* (D) and *Shh* (E). The telencephalic primordia is indicated in the drawing in F. The change in gene expression that takes place during expansion can be seen by comparing: A to Fig. 7B; B to Fig. 7D; C to Fig. 4H,J,L; D to Fig. 7F; E to Fig. 7K.

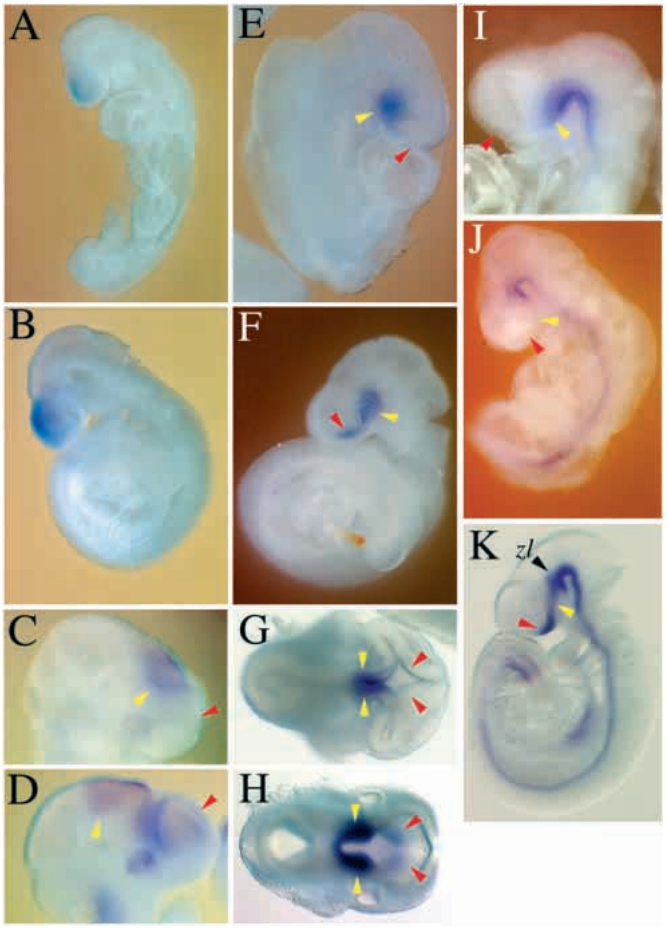

Fig. 7. Regionalization of the telencephalic primordium is defective in flat-top embryos. Whole-mount in situ hybridization of flat-top (A,C,E,G,I,J) and wild-type littermates (B,D,F,H,K) with markers of regional identity in the telencephalon. Yellow arrowheads indicate diencephalic domains of expression and red arrowheads indicate telencephalic domains. *Emx2* is expressed in the dorsal telencephalon of both wild-type and flat-top embryos (A,B; *n*=3). Telencephalic expression of *Pax6* is downregulated relative to expression in the diencephalon (C; *n*=5). Wild-type embryos show dorsally restricted expression at similar levels in both the diencephalon and the telencephalon (D). Expression of *Nkx2.1* is normal in the ventral diencephalon but is absent in the ventral telencephalon of flat-top embryos (E,G; *n*=6). In comparison, telencephalic expression is easily detectable in wild-type litter-mates (F,H). (G,H) Dorsal views of the heads of embryos. Expression of *Shh* is normal at 9.0 dpc (I; *n*=4) but is absent, not only from the telencephalon but also from all of the neurectoderm anterior to the zona limitans intrathalamica, at 10.5 dpc (J; *n*=4). The normal pattern of Shh expression anterior to the zona limitans (zl) is shown in K.

anterior to the zona limitans was specifically downregulated while more posterior expression remained (Fig. 7I-K).

DISCUSSION

Mutant screens in mice

The value of random mutagenesis and screens for specific adult phenotypes in mice was clearly demonstrated by the identification of *clock*, a locus that regulates circadian rhythms (Vitaterna et al., 1994). In spite of this success, direct screens for embryological phenotypes have been rarely attempted in mice (Kasarskis et al., 1998). Technical constraints have made the effort involved seem daunting. A significant impediment is the intrauterine development of placental mammals which limits access to the early stages of development. Screens must be carried out by dissecting embryos from the uterus at a defined stage of development. Substantial breeding is necessary because lethality associated with mutations in developmentally important genes dictates the isolation of recessive alleles. Despite these constraints, two recent advances have made embryological screens in mice more attractive. The first has been the recognition of ENU as a highly effective mutagen with a per locus mutation rate of approximately 1 in 700. Thus, the use of ENU substantially reduces the number of animals that must be examined to find relevant phenotypes. Identification of flat-top and four other mutants with related phenotypes involved a relatively modest effort that nonetheless screened through an estimated 5-10% of the genes in the genome. Improvements in our ability to detect subtle alterations in development at this early stage would expand the yield from such a screen. The second advance has been the rapid progression of technologies for positional cloning in mice. This has dramatically improved the feasibility of identifying mutated genes in mice and increased the value of mutants.

The role of the *flat-top* gene

Any model of *flat-top* gene action must explain three distinct forebrain defects. The first two defects are coupled: failure of the telencephalic primordium to expand and defects in telencephalic regionalization. The third defect becomes apparent

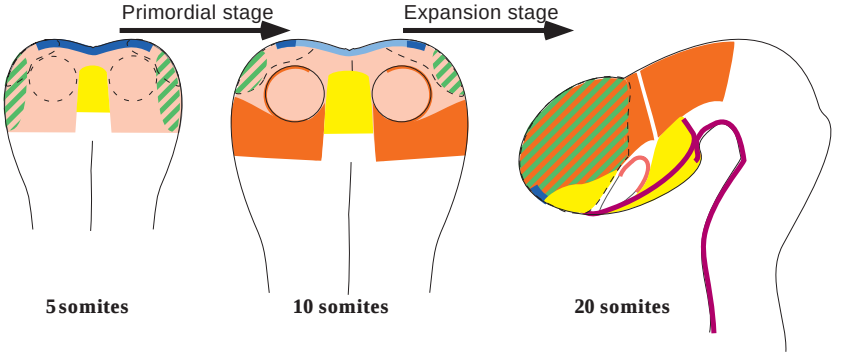

Fig. 8. Stages in the development of the telencephalon. The 10-somite stage is shown schematically and flattened although the neural folds are nearly touching but not yet closed at this stage. The telencephalon and its primordia are enclosed by dotted lines. Gene expression patterns: blue, *fgf8*; red, *Pax6*; pink, low level *Pax6*; yellow, *Nkx2.1*; purple, *Shh*; green stripes, *Emx2*. Anterior is at the top for the 5- and 10-somite stages and to the left for the 20-somite embryo.

later as mutant embryos lose the forebrain expression domains of *Shh* and BF-1 that had previously been established. The loss of BF-1 expression invites comparison to the phenotype of the BF-1 knockout mouse. In both cases regionalization and growth of the telencephalon are severely affected. The two genes are required at distinct stages in the development of the telencephalon however. The flat-top mutation affects an early stage, formation of the telencephalic vesicles. Vesicle formation occurs in BF-1 mutant embryos on the contrary, but the vesicles fail to develop further (Xuan et al., 1995).

The most upstream event in the development of the telencephalon that has been identified is the establishment of *Fgf8* expression at the anterior margin of the neurectoderm. *Fgf8* expression is not affected by the flat-top mutation suggesting that the *flat-top* gene acts either downstream of this inductive signal or in concert with it. The only known response to FGF8 in the forebrain is the induction of BF-1. Other targets are likely to exist and it is possible that *flat-top* is directly downstream of *Fgf8*. This straightforward interpretation is unlikely though, because the flat-top mutation interferes with the maintenance of *Shh* expression throughout the anterior forebrain. Based on the extent of the defects an alternative model would be that the *flat-top* gene affects all of the anterior neurectoderm by altering the regulation of forebrain gene expression. Forebrain development is regulated by external signaling centers, the node, the prechordal plate, the anterior visceral endoderm and the anterior neural ridge, and it is quite likely that the *flat-top* gene is required outside the neurectoderm, in one or more of these sites.

Relationship between turning and forebrain defects

The first defect to appear in flat-top embryos is a failure to turn. Wild-type embryos invariably rotate clockwise around the body axis and defects in embryonic turning are often associated with defects in the establishment of the left-right body axis. An association between dorsal-anterior development and left-right axis formation has previously been shown in both *Xenopus* and zebrafish. Treatments that disrupt dorsal-anterior development in the early *Xenopus* embryo also block the formation of the left-right axis. (Danos and Yost, 1995) Similarly, zebrafish mutations that prevent the formation of a notochord also interfere with left-right axis formation (Danos and Yost, 1996). The telencephalon is one of the most dorsal and one of the most anterior structures in the embryo and the coupling of turning defects with telencephalic defects in flat-top embryos may reflect a similar association. If the flat-top mutation does affect left-right axis formation the effect is incomplete. An early indicator of left-right asymmetry in vertebrate embryos is the rightward looping of the heart tube. Heart development proceeds normally in flat-top embryos indicating that asymmetric signals are present.

The role of proliferation in the flat-top phenotype

The development of flat-top embryos is delayed by approximately 4-8 hours relative to their littermates. Even when comparison is made on the basis of somite number, flat-top embryos are almost a factor of two smaller at 9.5-10.5 dpc. This size difference is likely to be the result of a slight reduction in the overall rate of cell division in flat-top embryos. Despite this growth deficit, structures whose formation is dependent upon cell division, such as the limb buds, develop normally. One explanation for the failure of telencephalic evagination is that the telencephalon is uniquely sensitive to a general defect in cell proliferation. An alternative explanation is that the failure of the telencephalon to develop properly reflects its particular sensitivity to patterning events regulated by the *flat-top* gene. Proliferation in the early mammalian embryo is intimately related to patterning and precedent for the latter view comes from studies of a number of different mutations in mice. For example, embryos that fail to express nodal, a secreted signaling molecule, in the visceral endoderm develop with severe anterior defects and similar or more severe growth deficits (Varlet et al., 1997). Embryos that develop without a morphological node as the result of a mutation in the transcription factor gene HNF3β have both obvious patterning defects and size deficits (Ang and Rossant, 1994; Weinstein et al., 1994). The most striking similarities are between flat-top embryos and embryos that have mutations in both the gsc and HNF3β transcription factor genes ($gsc^{-/-}$; $HNF3\beta^{+/-}$ embryos) (Filosa et al., 1997). HNF3β-gsc compound mutants are growth retarded and have specifc defects in development of the forebrain. As is the case for flat-top, BF-1 expression is found initially in the ventral portion but not the dorsal portion of the telencephalon. Also similar to the case of flat-top, the initial expression of BF-1 is lost soon after its initial induction.

The earliest stages of telencephalic development

Characterization of the flat-top phenotype has helped to more clearly define two discrete stages in the early development of the telencephalon. It has also established a relationship between expansion and regionalization of the telencephalon.

An initial stage in the development of the telencephalon is the establishment of paired primordia in the neuroepithelium dorsal and anterior to the optic vesicles. This is followed by a stage during which the primordia undergo rapid expansion and evagination to form the vesicles (Fig. 8). In the mouse embryo, the primoridia are relatively small regions that are not morphologically obvious. The *BF-1* gene is expressed in the primordia however and this has been used to uncover some aspects of the first stage in telencephalic development. BF-1 expression is induced in the neural plate by FGF8 suggesting that this signaling molecule is a key element in the establishment of the telencephalic primordia. *Fgf8* expression is remodeled during the primordial stage although the significance of this has not been established. *Fgf8* is initially expressed across the anterior edge of the neural plate in the ANR. A few hours after this initial expression domain is established two lateral domains appear and ANR expression itself is downregulated. Some regionalization of the primordia takes place before the end of this stage; *Emx2* expression begins during the primordial stage where it appears to mark the future dorsal telencephalon. The next stage of telencephalic development, progression from the primordia to the vesicles, is marked by closure of the neural tube. Closure brings the two lateral domains of *Fgf8* expression together to form the commissural plate. Expansion of the primordia then begins. At the same time, additional regionally restricted gene expression begins; a gradient of BF-1 expression becomes apparent, *Pax6* is dramatically upregulated and discrete telencephalic domains of *Shh* and *Nkx2.1* appear that were undetectable in the primordium.

The flat-top mutation disrupts both the expansion and regionalization that takes place during the second stage of telencephalic development, suggesting that these two processes are coupled. This may be because both processes are regulated by the same mechanism. For example a single signaling center

may produce signals that pattern and regulate expansion. Alternatively expansion may depend upon regionalization or vice versa. Resolution of these issues will require identification of the *flat-top* gene and determination of its sites of action in the developing embryo.

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