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Wnt and Bmp signalling cooperatively regulate graded *Emx2* expression in the dorsal telencephalon

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SUMMARY

Pattern formation of the dorsal telencephalon is governed by a regionalisation process that leads to the formation of distinct domains, including the future hippocampus and neocortex. Recent studies have implicated signalling proteins of the Wnt and Bmp gene families as well as several transcription factors, including *Gli*3 and the Emx homeobox genes, in the molecular control of this process. The regulatory relationships between these genes, however, remain largely unknown. We have used transgenic analysis to investigate the upstream mechanisms for regulation of *Emx*2 in the dorsal telencephalon. We have identified an enhancer from the mouse *Emx*2 gene that drives specific expression of a *lacZ* reporter gene in the dorsal telencephalon. This element contains binding sites for Tcf and Smad proteins, transcriptional mediators of the Wnt and Bmp signalling pathway, respectively. Mutations of these binding sites abolish telencephalic enhancer activity, while ectopic expression of these signalling pathways leads to ectopic activation of the enhancer. These results establish *Emx*2 as a direct transcriptional target of Wnt and Bmp signalling and provide insights into a genetic hierarchy involving *Gli*3, *Emx*2 and Bmp and Wnt genes in the control of dorsal telencephalic development.

Key words: Transgenic mice, *Gli*3, Wnt, Bmp, *Emx*, Forebrain

INTRODUCTION

The embryonic dorsal telencephalon of vertebrates gives rise to the cerebral cortex, which, as the seat of consciousness and higher cognition, constitutes the most complex and divergent structure in the CNS. Based upon histological and functional properties, the cerebral cortex is divided into many distinct domains such as the six-layered isocortex (neocortex) and the non-six-layered allocortices, which include the archicortex (hippocampus) and the paleocortex, as well as transitional cortices (Zilles and Wree, 1995). The neocortex and the allocortex form further subdomains that acquire specific cytoarchitectural and connectional characteristics. A fundamental issue in developmental neurobiology concerns the patterning mechanisms by which these different areas are generated. It is thought that telencephalic development comprises a multistep process controlled by a complex genetic program (for a review, see Wilson and Rubenstein, 2000). Early patterning of the dorsal telencephalon requires a regionalisation process, which leads to the generation of specific areas such as neocortex and hippocampus.

The molecular mechanisms that underlie the specification of these domains are just beginning to be deciphered. Inductive interactions are fundamental to the formation of all brain structures and several recent studies provide evidence for the involvement of signalling molecules of the Bmp and Wnt gene families in patterning the dorsal telencephalon. Explant cultures and in vivo misexpression suggest a role for Bmp proteins in the regulation of cell survival and proliferation (Furuta et al., 1997; Golden et al., 1999). In addition, mutant analysis has demonstrated a requirement for Wnt signalling in the specification of hippocampal cell fates, as *Wnt*3a mutant embryos lack the hippocampus (Lee et al., 2000). Similar defects have been observed in mice mutant for *Lef1* (Galceran et al., 2000), which encodes a transcriptional regulator of the Wnt signalling pathway and is therefore likely to mediate the Wnt3a effects on hippocampal development. Despite these recent advances in the identification and characterisation of these inductive signals involved in dorsal telencephalic development, the genetic targets controlled by Wnt/Bmp signalling remain elusive.

In addition to these inductive signals, the homeobox genes *Pax*6 and *Emx*2 have been implicated in the specification of cortical subdomains. Both genes are expressed throughout the dorsal telencephalon and control multiple aspects of its development. *Emx*2 homozygous null mutant embryos have a reduced hippocampus and neocortex (Pellegrini et al., 1996; Tole et al., 2000a; Yoshida et al., 1997) that also display neuronal migration defects due to impaired reelin signalling (Mallamaci et al., 2000a). Loss of *Pax*6 function leads to altered dorsoventral patterning in the telencephalon (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001), and results in disruption of radial glia fascicles (Götz et al., 1998) and in defective migration of cortical precursors (Caric et al., 1997). Interestingly, the *Emx*2 and *Pax*6 expression patterns form opposing gradients along the rostral/caudal and medial/lateral

axes of the dorsal telencephalon. The graded and opposing activities of these homeodomain transcription factors is thought to confer regional identity to cortical subdomains as loss-of-function mutation of either gene leads to an expansion of specific areas at the expense of others (Bishop et al., 2000; Mallamaci et al., 2000b). Given the significance of graded *Emx*2 and *Pax*6 expression for the generation of cortical subdomains, it will be important to identify the molecular mechanisms that establish these expression patterns.

The extra-toes mouse mutant (Xt^J) which is defective for the zinc finger transcription factor *Gli*3 (Büscher et al., 1998) provides an excellent mouse model with which to study the molecular mechanisms leading to the specification of distinct cortical domains. Xt^J homozygous embryos lack the hippocampus at all developmental stages and display defects in cortical specification (Franz, 1994; Theil et al., 1999). On a molecular level, these defects correlate with a loss of Bmp and of some Wnt gene expression, and with a severe reduction and/or loss of *Emx*1/2 transcription from the dorsal telencephalon (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000b). These altered expression patterns, as well as the similarities in the phenotypes of *Gli*3, *Emx*2 and *Wnt*3a mutant mice, suggest that these genes form part of a genetic cascade that controls dorsal telencephalic development. To begin to elucidate the regulatory relationship between these genes, we analysed the transcriptional regulation of *Emx*2 in transgenic mice. We identified an *Emx*2 enhancer that was capable of driving expression of a *lac*Z reporter gene in the telencephalon and we show that Bmps and Wnts synergistically act on this enhancer through Smad and Tcf transcription factors. These data provide an important insight into the molecular mechanisms that lead to graded *Emx*2 expression and thereby to the specification of cortical domains.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

For transgenic analysis, fragments from an *Emx*2 P1 clone were subcloned into an end-filled *Sal*I site of the *lac*Z reporter vector pGZ40 upstream of the human β-globin promoter (Yee and Rigby, 1993). The constructs contained the following genomic fragments: constructs 1 and 2, 4.6 kb *Sac*II-*Hin*dIII in opposite orientations; construct 3, 1.6 kb *Sac*II-*Xho*I; construct 4, 2.9 kb *Nde*I-*Sac*II; construct 5, 3.4 kb *Xho*I-*Hin*dIII; construct 6, 2.2 kb *Hin*dIII-*Nsi*I; construct 8, 1.7 kb *Hin*dIII-*Nde*I; construct 9, 2.5 kb *Eco*RI-*Bam*HI, construct 10, 1.5 kb *Bgl*II-*Nsi*I. For the generation of construct 7, 200 bp of the *Emx*2 enhancer were PCR amplified with the oligonucleotides 5′AAACTCGAGTGGGTCTGCAATGCTGTGAC3′ and 5′GAGGTGAGGGATCAAGTAAG3′. The *Nde*I/*Xho*I digested PCR product was subcloned together with an 1.5 kb *Hin*dIII/*Nde*I fragment into pGZ40 cut with *Xho*I/*Hin*dIII. To generate construct 11, the 0.2 kb *Xho*I/*Nde*I (blunt) fragment from construct 7 was subcloned into construct 2 digested with *Xho*I and *Bgl*II (blunt). For the generation of transgenic mice, fragments were either purified after a *Bgl*II/*Sac*II digest of construct 8 (construct 12) or after a *Kpn*I/*Sac*II digest (all other constructs). Site-directed mutagenesis reactions of the Tcf- and Smad-binding sites were performed on the 0.4 kb *Hin*dIII-*Bgl*II fragment subcloned into the pBS-KS vector. The oligonucleotides used were: 5′TCTGTTCGAGCGCCAAAGCGTCCT3′ and 5′ACAGACGAGGTTTCTCTATCA3′ (Tcf-binding site); 5′GCTCGAACAGAACACACGAGGTTTCTC3′ and 5′TACAAAGCGTCCTGTGAGCTT3′ (Labbe et al., 2000). After sequencing, a 0.2 kb *Sph*I/*Stu*I fragment from construct 2 was replaced by a corresponding fragment containing the desired mutation.

Generation and analysis of transgenic mice

Transgenic mice were generated by microinjection of fertilised eggs from crosses between F_1 hybrids (C57Bl6×C3H) as described previously (Theil et al., 1998) and were identified by polymerase chain reaction using extra-embryonic yolk sac or tail DNA. Expression of the transgene was analysed by staining mouse embryos for β-galactosidase activity (Theil et al., 1998).

Xt^J mutant mice were kept as heterozygous animals in a mixed C57Bl6/C3H background and were interbred. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. Xt^J/Xt^J embryos were readily distinguished from heterozygous and wild-type embryos by forebrain morphology (Johnson, 1967).

Whole-mount in situ hybridisation

In situ hybridisation of whole-mount mouse embryos was performed as described (Theil et al., 1999) using riboprobes for the following genes: *Wnt*3a (Roelink and Nusse, 1991), *Wnt*8b (Richardson et al., 1999) and *Wnt*7b (Parr et al., 1993).

Electrophoretic mobility shift assay

pGEX4T-1/Lef1 amino acids 1-397 was a kind gift from Rolf Kemler. The Smad1MH1-coding region (amino acids 1-167) was PCR amplified using the oligonucleotides 5′AATGGATCCATGAATGTGACCAGCTTGTTTTC3′ and 5′AATGGATCCGGCATGTGAGGCTCATTTTGTC3′ and subcloned into the *Bam*HI site of pGEX4T-1. Recombinant GST fusion proteins were prepared as described (Arnold et al., 2000).

For electrophoretic mobility assays, the following oligonucleotides covering the wild-type or mutated Tcf- and Smad-binding sites were used: 5′CACAGGACGCTTTGTAGCTCGAACAGAACAGACGAGGTTTCTC3′. In the mutated forms, the underlined bases were replaced by GC and C, respectively. GST-Lef1 (100 ng) and GST-Smad1MH1 (300 ng) fusion proteins were incubated for 30 minutes on ice in 20 mM Hepes, pH 7.9; 60 mM KCl; 1 mM EDTA, pH 8.0; 1 mM DTT, 5 mM $MgCl_2$, 10% glycerol and 1 μg poly(dI-dC) in the presence of 10,000 cpm of the end-labelled oligonucleotides. Electrophoresis was performed through 5% native polyacrylamide gels in 0.5×TBE at room temperature.

Electroporation and explant culture of mesencephalic neuroectoderm

The mesencephalon of E11.5 embryos was dissected and the surface ectoderm removed manually. The resulting tissue was placed in a DNA solution containing construct 2 as a *lac*Z reporter plasmid either alone or in combination with plasmids coding for constitutive active forms of β-catenin (Aberle et al., 1997) and ALK3 receptor (Kawai et al., 2000) each at 1 mg/ml. For electroporation, electrodes were placed in contact with the dissected mesencephalon and three pulses at 70 V each for 50 mseconds were delivered. Electroporated tissue pieces were cultured on a micropore filter (Costar, #110414) residing on a metal grid in organ culture dishes (Falcon, #3037). The culture medium was Dulbeccco's Modified Eagle's Medium (DMEM) supplemented with 20% foetal bovine serum, 1× non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco) and 1× streptomycin/penicillin (Gibco). After 6 hours in culture, explants were processed for immunohistochemistry or for β-galactosidase staining as described above for embryos.

Immunohistochemistry

Immunohistochemistry was performed on dissected tissue pieces after electroporation and in vitro culture. After fixation in 4% PFA for 2 hours, the tissue was pre-incubated in a solution of 1% normal goat serum in PBST (PBS with 0.1% Triton X-100). After 2 hours, the liquid was changed for a fresh aliquot containing the primary antibody

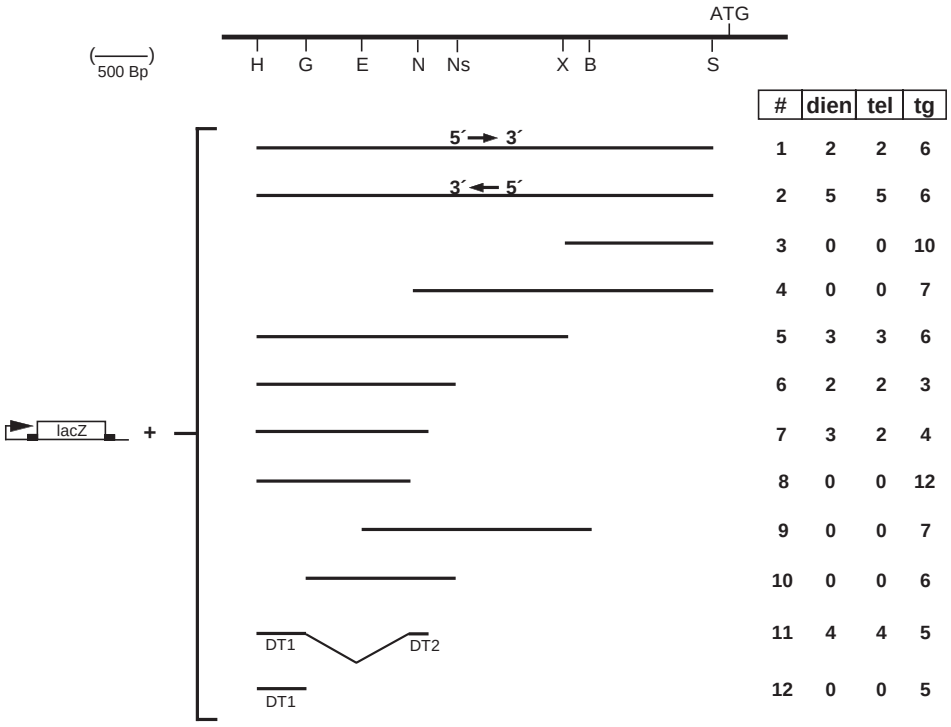

Fig. 1. Transgenic analysis of *Emx*2 regulation in the forebrain. The diagram depicts a restriction map upstream of the *Emx*2-coding region (the translational start (ATG) is indicated). The restriction fragments, which are cloned into a vector containing *lac*Z and a minimal promoter are represented by bars. For each construct, the numbers of transgenic embryos that showed β-galactosidase staining in the telencephalon (tel) or diencephalon (dien) and the total number of transgenic embryos (tg) are indicated. B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nde*I; Ns, *Nsi*I; S, *Sac*II; X, *Xho*I.

[anti-Myc (mouse monoclonal, 1:200; Santa Cruz)] and the tissue was incubated at 4°C overnight. The tissues were washed three times in PBST and the secondary antibody [1:200 donkey anti-mouse IgG, conjugated to Cy3 (Jackson ImmunoResearch)] was applied for 4 hours at room temperature. Fluorescence imaging was carried out on a Leica TCS NT confocal microscope. GFP signal was imaged using the standard FITC filter. Images were processed and mounted using Photoshop 6.0 (Adobe).

RESULTS

Identification of an *Emx*2 enhancer

To begin to unravel the regulatory relationships between *Gli*3, *Emx*2, Bmp and Wnt genes, we started to investigate the *cis* requirements for *Emx*2 expression, which occurs in a dynamic pattern in the mouse forebrain (Simeone et al., 1992; Gulisano et al., 1996). Initially, *Emx*2 mRNA can be detected in anterior dorsal neuroectoderm between E8.5 and E9.0. By E9.5, the expression has been restricted to a domain extending between the olfactory placodes and the roof of the rostral diencephalon. During development of the cerebral cortex, *Emx*2 expression is confined to the neuroepithelium forming an expression gradient along the anterior/posterior and medial/lateral axis from E12.0 with highest expression levels in the caudal/medial domain. Additional expression sites in the developing forebrain are observed in specific areas of the dorsal, medial and ventral diencephalon (Simeone et al., 1992; Gulisano et al., 1996).

To elucidate the transcriptional regulation underlying this expression pattern, genomic fragments from the mouse *Emx*2 gene were linked to a *lacZ* reporter gene under the control of the human β-globin minimal promoter and tested for enhancer activity in transgenic mice. Fig. 1 depicts the mouse *Emx*2 genomic region surrounding the first coding exon and summarises the regulatory regions examined and their enhancer activity. Initially, we identified a 4.6 kb fragment immediately upstream of the *Emx*2 translational start site that mediates dorsal telencephalic expression at E10.5 in a manner analogous to the endogenous *Emx*2 gene (compare Fig. 2A with 2B). Identical expression patterns were obtained in either orientation of this fragment relative to the minimal promoter (constructs 1 and 2 in Fig. 1). Unlike the endogenous gene, however, stronger and more widespread reporter gene activity occurred in the ventral diencephalon (Fig. 1, Fig. 2A,B) (Simeone et al., 1992), suggesting that not all elements are included in this construct that normally regulate transcription in this tissue. Thus, regulatory elements in the 4.6 kb fragment are sufficient to drive expression of a transgene in specific sites of *Emx*2 gene expression, and show characteristics of enhancers as they work on an heterologous promoter and in an orientation-independent manner.

To characterise these regulatory elements further, three independent transgenic lines were established carrying the 4.6 kb fragment linked to the reporter gene and were analysed for β-galactosidase activity at various developmental stages. Identical results were obtained from these lines. In contrast to the endogenous gene, reporter gene activity was first detected in the seven somite stage in the ventral forebrain, which, based upon the staining pattern at E10.5, prefigures expression in the ventral diencephalon (data not shown). At the 12-somite stage, β-galactosidase staining extends into the dorsal forebrain (Fig. 2D) to eventually cover the dorsal telencephalic vesicles at E9.5 with highest expression levels in the caudal most part of the telencephalon (Fig. 2E). Although at E10.5 *lac*Z expression occurred in the complete dorsal telencephalon at uniform levels (Fig. 2B), reporter gene expression appeared at higher expression levels in the caudal/medial telencephalon at E11.5, probably reflecting the establishment of the *Emx*2 expression gradient (Fig. 2F). These data indicate that the 4.6 kb enhancer is sufficient to confer reporter gene expression in the dorsal

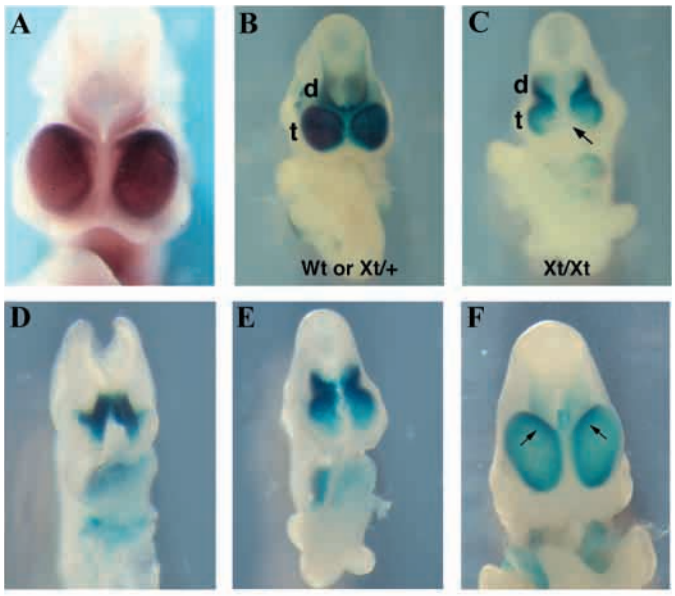

Fig. 2. Characterisation of the *Emx*2 enhancer activity in the forebrain. (A) In situ hybridisation analysis of *Emx*2 expression in the telencephalon of E10.5 wild-type mice. (B) *lac*Z staining of a transgenic embryo carrying the mouse *Emx*2 enhancer (construct 2). (C) *Emx*2 enhancer activity in the forebrain of an homozygous Xt^J embryo. *lac*Z expression in the dorsomedial telencephalon (t) is abolished (arrow), while expression in the cortical neuroepithelium occurs at lower levels than in wild-type embryos. *lac*Z expression in the diencephalon (d) remains unaffected. (D-F) Time course analysis of enhancer activity in the forebrain at (D) the 12-somite stage, (E) E10.5 and (F) E11.5. (D) Start of *lac*Z expression in the dorsal forebrain. (E) Expression of the reporter gene is observed in the dorsal part of the telencephalic vesicles with highest expression levels in the caudal telencephalon. (F) At E11.5, the reporter gene is expressed in a gradient with highest expression levels in the medial-caudal telencephalon (arrows). *lac*Z expression in the developing heart as seen in B-F is specific for the transgenic line shown.

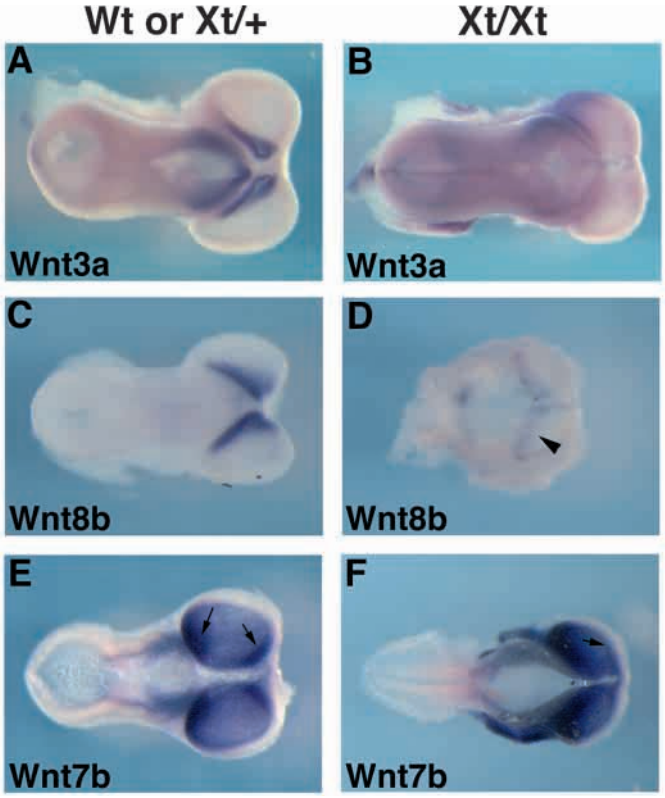

Fig. 3. In situ hybridisation analysis of Wnt gene expression in the forebrains of E10.5 embryos lacking Gli3 function. (A,B) *Wnt*3a expression in wild-type embryos covers the dorsomedial telencephalon, while Xt^J/Xt^J embryos completely lack *Wnt*3a mRNA in this tissue. (C,D) *Wnt*8b expression in homozygous Xt^J embryos is disrupted, except for a narrow band of cells in the caudal telencephalon (arrowhead) (E) *Wnt*7b is expressed in the dorsal telencephalon of wild-type embryos with highest expression levels in the dorsomedial part (arrows). (F) *Wnt*3a mRNA is specifically absent from the dorsomedial telencephalon of Xt^J/Xt^J embryos (arrow), but still present in the dorsolateral telencephalic neuroepithelium.

telencephalon, but does not reflect the early phases of *Emx*2 expression in the forebrain.

Using deletion analysis on the 4.6 kb fragment, we aimed to identify minimal elements involved in dorsal telencephalic expression. Using this approach, we mapped regulatory activities to two, 450 and 180 bp, fragments (DT1 and DT2, respectively) at the 5′ end of construct 1 (Fig. 1). All constructs containing these two elements showed enhancer activity in the telencephalon and diencephalon. Deletion of either element resulted in loss of reporter gene expression in the forebrain (construct 8 and 10). Similarly, a construct containing just DT1 did not show enhancer activity in the dorsal telencephalon (construct 12). By contrast, a construct in which DT1 is fused to DT2 drove *lacZ* expression in the dorsal forebrain in a pattern indistinguishable from the original 4.6 kb enhancer (construct 11). Both, DT1 and DT2 are therefore necessary and, in combination, sufficient components of an enhancer-driving region-specific expression of a reporter gene in the developing forebrain of transgenic mice.

We have previously shown that *Emx*2 expression is severely reduced in extra-toes (Xt^J) mutant mice (Theil et al., 1999). To address whether enhancer activity is similarly affected by the *Gli3* mutation, we generated transgenic lines from construct 2 and compared transgene expression in wild-type/heterozygous and homozygous Xt^J genetic backgrounds. Similar to the endogenous *Emx*2 gene, *lacZ* expression was completely abolished from the dorsomedial telencephalon of Xt^J/Xt^J embryos (Fig. 2B,C). Interestingly, however, the dorsolateral telencephalon still showed enhancer activity albeit at reduced levels. An identical expression pattern was observed at E11.5 in Xt^J/Xt^J embryos (data not shown), indicating that these alterations do not reflect a delay in enhancer activity.

The *Emx*2 forebrain enhancer contains binding sites for Tcf and Smad transcription factors

The localisation of the *Emx*2 forebrain enhancer activity to DT1 and DT2 enabled us to specifically search for potential upstream factors that mediate this activity. Loss of *Emx*2 expression in Xt^J/Xt^J embryos (Theil et al., 1999; Tole et al., 2000b) suggested a potential direct regulation of *Emx*2 by Gli3. However, the absence of any obvious consensus Gli-binding site within these elements and even in the complete 4.6 kb fragment argues against this possibility and favours an indirect regulatory fashion. Other potential direct upstream candidate

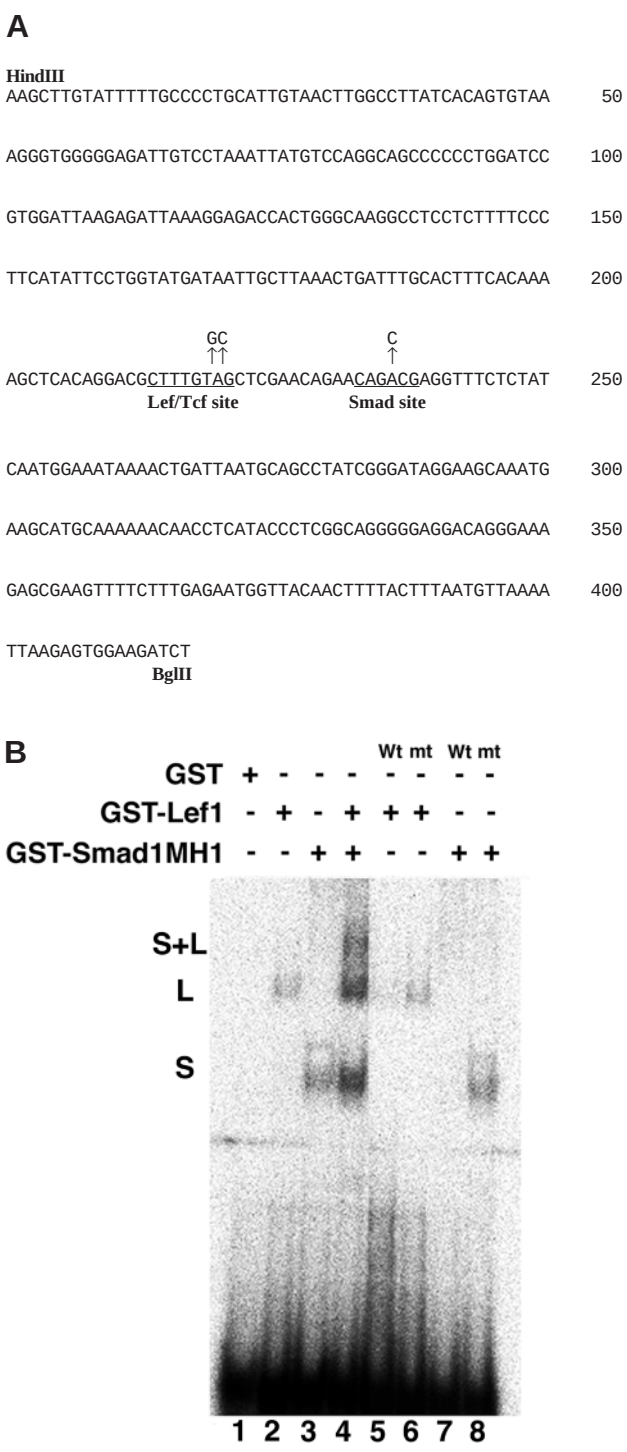

Fig. 4. The DT1 element of the *Emx*2 enhancer contains adjacent Tcf- and Smad-binding sites (A) Nucleotide sequence of DT1. The canonical Tcf- and Smad-binding sites (underlined) are indicated. For mutational analysis, the underlined nucleotides were replaced by GC and C, respectively. (B) The binding capacity of the *Emx*2 enhancer was tested in electrophoretic mobility shift assays with recombinant Lef1 and Smad1MH1 protein. Lef1 and Smad1 bind to an *Emx*2 enhancer oligonucleotide (lanes 2 and 3, respectively). Binding is potentiated in the presence of both proteins (lane 4) and an additional slower migrating, Lef1/Smad1 complex is observed. Complex formation is competed by a molar excess (100×) of the *Emx*2 enhancer oligonucleotide but not by an oligonucleotide containing mutations in the Tcf- and Smad-binding sites (lanes 5-8).

regulators include members of the Bmp and Wnt gene families which control important aspects of dorsal telencephalic development. To determine whether these genes could play a role in *Emx*2 regulation, we monitored their expression patterns in the telencephalon of homozygous Xt^J embryos at E10.5. Given the severe reduction of *Emx*2 expression in these embryos and the possible involvement of Bmps and Wnts in *Emx*2 regulation, the expression of all Bmps and Wnts should also be affected by the loss of *Gli*3 activity. The expression of all Bmps in the telencephalon of homozygous Xt^J embryos is lost or severely reduced (Theil et al., 1999; Tole et al., 2000b). In addition, some Wnt genes are expressed in nested domains during early cortical development (Lee et al., 2000). As reported previously for E12.5 (Grove et al., 1998), *Wnt*3a expression is already lost from the telencephalon of E10.5 Xt^J homozygous embryos (compare Fig. 3A,B). Although *Wnt*8b expression has been reported to be unaffected in Xt^J/Xt^J embryos at E12.5 (Tole et al., 2000b), we were not able to detect *Wnt*8b mRNA in the telencephalon of E10.5 homozygous Xt^J embryos except for a band of weak expressing cells in the caudal cortical neuroepithelium (Fig. 3C,D). By contrast, absence of functional *Gli*3 did not abolish *Wnt7b* transcription in the dorsolateral telencephalon whereas the high expression level domains in the medial telencephalon were lost (Fig. 3E,F). Thus, Xt^J homozygous embryos lack the medial expression domain of all Wnt and Bmp genes in the telencephalon, while dorsolateral expression of *Wnt*7b persisted.

The absence of Bmp and Wnt gene expression from the dorsomedial telencephalon of *Gli*3 mutant embryos further suggested the possible involvement of the corresponding signalling pathways in *Emx*2 gene regulation. To address the possibility that transcription factors that mediate Bmp and Wnt signalling are direct transcriptional regulators, we searched for binding sites of these factors within DT1 and DT2. Transcriptional regulation of certain Wnt target genes occurs through the nuclear translocation of a transcriptional complex containing β-catenin and a member of the Tcf family (for a review, see Polakis, 2000). Similarly, transmitting the Bmp signal involves the activation of Smad1, Smad5 or Smad8 transcription factors (for a review, see Attisano and Wrana, 2000). Interestingly, examination of the *Emx*2 regulatory elements revealed the presence of a canonical Tcf-binding site immediately adjacent to a consensus Smad-binding site within DT1 (Fig. 4A). To determine whether Tcf and Smad proteins would bind to these motifs, we performed electrophoretic mobility shift assays using recombinant glutathione-S-transferase (GST) protein fused either to full-length Lef1 protein or to the Smad1 DNA-binding domain (Smad1MH1). Incubation of an oligonucleotide containing both binding sites with Lef1 fusion protein produced a protein/DNA complex with reduced electrophoretic mobility compared with DNA alone (Fig. 4B). In addition, in the presence of Smad1MH1 fusion protein, a DNA-binding complex was observed. The formation of both complexes was, however, enhanced when both fusion proteins were used. Moreover, an additional slower migrating, possibly secondary Lef1/SmadMH1 complex was

observed under these conditions. Blocking the formation of Lef1/DNA and Smad1MH1/DNA complexes was achieved with an excess of double-stranded oligonucleotide but not with surplus oligonucleotide containing mutated binding motifs demonstrating specific binding to the *Emx*2 enhancer sequences (Fig. 4B). Thus, Lef1- and Smad1MH1 fusion proteins are capable of specific binding to the *Emx*2 regulatory element in vitro.

Tcf- and Smad-binding sites are required for forebrain enhancer activity

The role of the Tcf/Smad-binding sites in regulating dorsal telencephalic *Emx*2 expression in vivo was determined by generating point mutations in these sites within the context of the 4.6 kb enhancer and testing the activity of the *lac*Z reporter gene in transgenic embryos. To minimise the modifications to the element, we introduced point mutations into each binding site (Fig. 4A), which have previously been shown to drastically reduce or even abolish binding of Tcf and Smad factors to their respective binding motifs (Zawel et al., 1998; Tetsu and McCormick, 1999). Mutations in both sites completely abolished *lac*Z staining in the dorsal telencephalon in seven out of eight independent transgenic embryos (Fig. 5B). By contrast, the ventral diencephalon still showed enhancer activity although at a severely reduced level (Fig. 5B). Only one transgenic embryo displayed weak β-galactosidase staining in the caudal-most telencephalon (Fig. 5C).

These data indicate that Tcf/Smad-binding sites are essential elements for the control of regional *Emx*2 expression in the telencephalon. Next, we determined the relative contributions of the individual sites to the enhancer activity in transgenic embryos. A construct with a mutated Tcf-binding site but with an intact Smad-binding site, resulted in weak enhancer activity in the dorsomedial telencephalon (six out of eight *lac*Z-positive embryos) consistent with *Bmp* expression and signalling being confined to this domain (Fig. 5D). By contrast, the Tcf-binding site alone was capable of directing weak *lac*Z expression to the cortical neuroepithelium and stronger expression in the dorsomedial telencephalon with a pattern highly reminiscent of *Wnt7b* expression (seven out of nine *lac*Z-positive embryos) (Fig. 5E; compare with Fig. 3E). Similar to the construct containing mutations in both Tcf- and Smad-binding sites, diencephalic *lac*Z expression was not abolished by the single site mutation but the expression level was severely reduced (Fig. 5D,E). These data indicate that only a subset of telencephalic cells displays *Emx*2 enhancer activity in the presence of either the Tcf-binding site or the Smad-binding site. Both binding sites together are therefore required for full enhancer activity in the telencephalon.

Transactivation of *Emx*2 by ectopic activation of the Wnt and Bmp signalling pathway

To obtain further evidence for the ability of the Wnt and Bmp signalling pathways to upregulate *Emx*2 expression through the telencephalic enhancer, we wanted to determine the consequences of ectopic activation of these pathways on enhancer activity. However, constitutive activation of these signalling pathways during embryogenesis might interfere with normal development, which would not allow us to analyse the effects on the *Emx*2 enhancer. We therefore tried to activate Wnt and Bmp signalling specifically at later time points of development using an electroporation assay combined with subsequent in vitro culture. For this purpose, the mesencephalon of E11.5 mouse embryos was dissected, which lacks *Emx*2 telencephalic enhancer activity. Special care was taken not to include the isthmic region of the midbrain as an endogenous source of Wnt and Bmp proteins. The dissected mesencephalic tissue was then electroporated with different DNA constructs. After electroporation, the tissue was maintained for 6 hours under in vitro culture conditions and then monitored for expression and/or effects of the electroporated constructs. Control experiments in which we electroporated an expression vector encoding green fluorescent protein (GFP) showed robust GFP expression in the dissected tissue (Fig. 6A). To further analyse whether a mixture of different plasmids are efficiently electroporated we co-transfected the GFP expression vector with a plasmid coding for a Myc-tagged form of the ALK3-Bmp receptor. This co-electroporation led to the appearance of Myc-immunoreactivity in GFP-positive cells (Fig. 6B), suggesting extensive co-transfection of the electroporated constructs. In all subsequent experiments, we co-electroporated the GFP expression vector to assess the efficiency of each electroporation. Only those explants that showed similar GFP expression levels as shown in Fig. 6A were used for further analysis.

Dissected mesencephalic tissue was then electroporated with

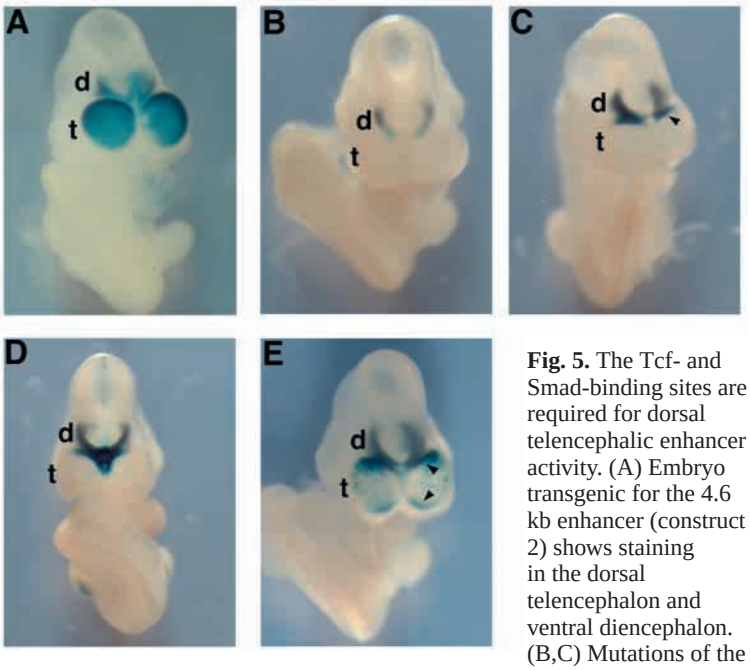

Fig. 5. The Tcf- and Smad-binding sites are required for dorsal telencephalic enhancer activity. (A) Embryo transgenic for the 4.6 kb enhancer (construct 2) shows staining in the dorsal telencephalon and ventral diencephalon. (B,C) Mutations of the Tcf- and Smad-binding sites lead to weak enhancer activity in the ventral diencephalon. *lac*Z staining in the dorsal telencephalon is completely abolished (B) or drastically reduced (arrowhead) (C). (D) Staining in embryos with a transgene carrying a mutation of the Tcf-binding site is restricted to the dorsomedial telencephalon. (E) Embryo carrying a transgene with a mutated Smad-binding site shows weak β-galactosidase activity throughout the cortical neuroepithelium with higher expression levels in the dorsomedial telencephalon (arrowheads).

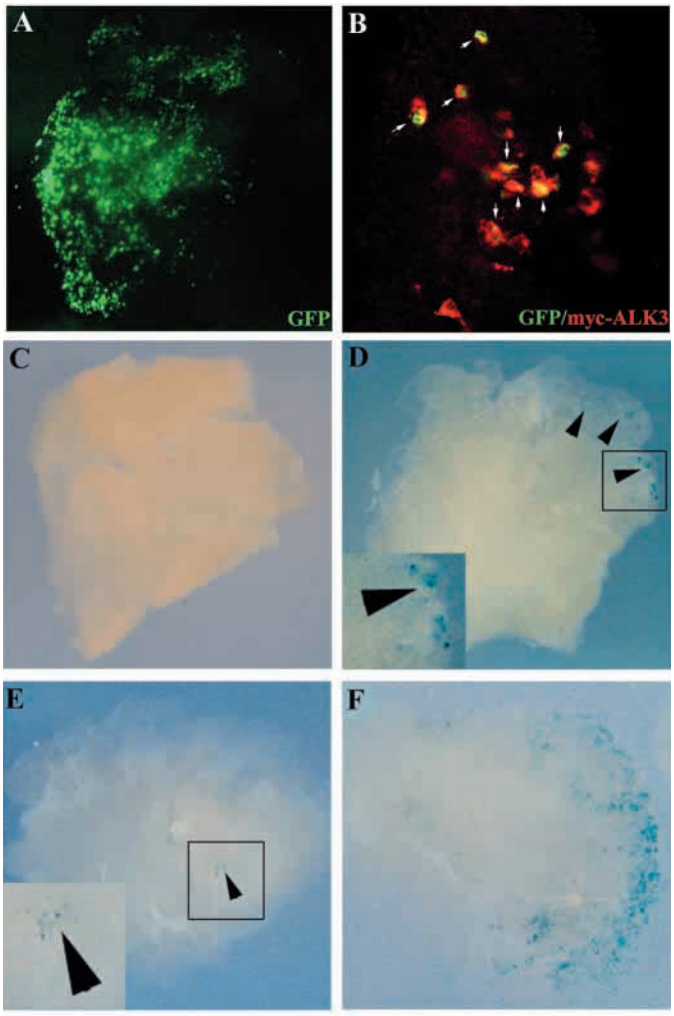

Fig. 6. Ectopic stimulation of the *Emx*2 enhancer through ectopic activation of Bmp and Wnt signalling. E11.5 mesencephalic tissue was electroporated with the indicated constructs and processed for immunohistochemistry (A,B) or stained for *lac*Z expression (C-F). (A) Electroporation of a GFP expression vector leads to robust GFP expression in dissected tissue. (B) Co-transfection of the GFP expression vector with a plasmid encoding a Myc-tagged Bmp receptor (ALK3 or Bmpr1a). Expression of both proteins, GFP and Myc-ALK3, is observed in the same cells (arrows). GFP expression was detected by its intrinsic green fluorescence while Myc-Alk3 protein expression was detected by immunofluorescent labelling with an anti-Myc antibody. (C) Electroporation of the reporter plasmid alone does not result in enhancer activity. Co-electroporation of the reporter gene construct together with either constitutive active β-catenin (D) or activated ALK3 (E) leads to an induction of enhancer activity in few cells (arrowheads). The inserts shown in D,E represent higher magnifications of the boxed areas. (F) The Emx2 enhancer is heavily stimulated after activation of both Bmp and Wnt signalling.

a DNA construct providing the *Emx*2 enhancer driving *lac*Z expression (construct 2 in Fig. 1) in combination with plasmids coding for constitutively active β-catenin (S33A mutant β-catenin (Aberle et al., 1997) and/or constitutive active Bmp receptor ALK3 (Kawai et al., 2000) under the control of a CMV promoter/enhancer. After electroporation, the mesencephalic tissue was maintained for 6 hours under in vitro culture conditions and then monitored for β-galactosidase staining. Whereas electroporation of the reporter plasmid alone did not result in enhancer activity (Fig. 6C) (n=10/10), ectopic activation of either the Wnt or the Bmp signalling pathway induced reporter gene expression in few cells (Fig. 6D,E) (n=9/12 and n=11/14 for constitutive active ALK3 and β-catenin, respectively). Moreover, the number of *lac*Z-positive cells massively increased after activation of both, the Wnt and Bmp pathways (Fig. 6F) (n=15/17). These experiments demonstrate, that Wnt and Bmp signalling act synergistically to stimulate reporter gene expression from the *Emx*2 telencephalic enhancer.

DISCUSSION

Emx2 is a direct Wnt and Bmp target gene

We have shown that a 4.6 kb genomic fragment immediately upstream of the *Emx*2-coding region confers region specific expression of a reporter gene in the dorsal telencephalon of transgenic mice. The corresponding regulatory elements act independent of the orientation of the 4.6 kb fragment, indicating that they possess characteristics of a transcriptional enhancer. Further analysis revealed two fragments, DT1 and DT2, that are both necessary and (in combination) sufficient for enhancer activity. The temporal and spatial coincidence of reporter gene expression with transcription of the endogenous *Emx*2 gene suggests that the genomic fragment contains all the necessary regulatory elements controlling this aspect of the expression of the gene in vivo.

Several lines of evidence strongly suggest that *Emx*2 expression in the dorsal telencephalon is directly regulated by Bmp and Wnt signalling in vivo. First, Tcf and Smad transcription factors, transcriptional mediators of these pathways, bind in vitro to sites within DT1 that are essential for enhancer activity in the telencephalon. Second, ectopic expression experiments indicate that ectopic activation of the Wnt and Bmp signalling pathway can stimulate *Emx*2 enhancer activity. Finally, genetic evidence has come from the analysis of a null mutant for the *Gli*3 gene showing that loss of Bmp and Wnt gene expression from the dorsal telencephalon coincides with a severe reduction of *Emx*2 expression (Theil et al., 1999; Tole et al., 2000b) (this paper). Although a direct regulation of *Emx*2 through Gli3 provides an explanation for these findings, this possibility seems unlikely because of the absence of any obvious Gli-binding site within the telencephalic enhancer. Furthermore, given the complex phenotype of *Gli3* mutant embryos, we cannot rule out the possibility that other not yet identified factors beside Wnts and Bmps are involved in *Emx*2 regulation. However, the findings on the altered expression patterns in Xt^J homozygous embryos taken together with the data presented here provide strong evidence that Wnts an Bmps are essential, immediate upstream regulators of *Emx*2.

Complexity of the regulation of *Emx2* expression

The analysis presented here has revealed several aspects of the complexity of *Emx*2 regulation. Although the 4.6 kb fragment mediates reporter gene expression in the dorsal telencephalon indistinguishable from the expression pattern of the

endogenous gene, enhancer activity was not observed in the early developing dorsal forebrain. This difference suggests that the spatial and temporal control of *Emx*2 expression might involve the use of distinct regulatory modules. A similar conclusion was obtained for the control of the segmental expression of the *Epha4* gene (Theil et al., 1998) and of the Hox genes in the hindbrain (Gould et al., 1998). A more detailed study will be required for the identification of those regulatory elements that control the early aspects of *Emx*2 expression in the forebrain.

A further difference between regulation of the endogenous and the reporter gene was observed in Xt^J homozygous embryos. While this mutation leads to a severe reduction of *Emx*2 expression in the neocortical neuroepithelium, enhancer activity is still detectable in this tissue. This discrepancy might be explained by different sensitivities of the detection methods. Furthermore, a Gli3 activating function might be required for high level *Emx*2 expression in the cortical neuroepithelium. However, Gli3 has only been observed as a transcriptional activator as a result of a Sonic hedgehog-induced proteolytical processing. Alternatively, *Gli*3 might negatively control the transcription and/or the activity of a repressor that would act on sequences outside of the 4.6 kb enhancer. According to this model, loss of *Gli*3 function would lead to a derepression of this repressor that, in turn, would suppress endogenous *Emx*2 expression but not transcription of the reporter gene as the 4.6 kb fragment lacks the necessary binding sites. Such a role for Gli3 correlates with its known repressor activities (Wang et al., 2000).

Several observations of our study indicate a cooperative interaction between Wnt and Bmp signalling to regulate *Emx*2 expression in the telencephalon. While mutations of the Tcf- and Smad-binding sites abolish *Emx*2 enhancer activity in the telencephalon, the single site mutations only affect specific aspects of reporter gene expression. Furthermore, in vitro binding of the Tcf/Smad factors is enhanced in the presence of both factors. Similarly, our ectopic expression experiments show an increased induction of the telencephalic enhancer through both, Wnt and Bmp signalling. Synergy between Tgfβ and Wnt signalling to regulate developmental events has been observed in various cases (Cadigan and Nusse, 1997; Whitman, 1998) and may involve direct interactions between Lef1 and Smad proteins (Labbe et al., 2000; Nishita et al., 2000). As expression of Bmp family members is confined to the dorsomedial telencephalon, a cooperative effect between Wnt and Bmp signalling would mainly be restricted to development of the hippocampus and adjacent medial neocortex. Interaction between these signalling pathways therefore provides a molecular mechanism to specify the gradient of *Emx*2 expression along the medial/lateral axis of the telencephalon (see later).

Within the neocortical neuroepithelium, control of regional *Emx*2 expression requires a functional Tcf-binding site. The similarities between the *Wnt*7b expression and the β-galactosidase staining pattern of the *Emx*2 enhancer construct just containing the functional Tcf-binding site make this *Wnt* family member a good candidate for being an upstream regulator of *Emx*2 expression in the telencephalon. This idea is further supported by recent findings showing that Wnt7b can induce the formation of a free cytoplasmic pool of β-catenin (Arnold et al., 2000) and can stimulate the expression of the Tcf target gene *Cdx1* (Lickert et al., 2000). In addition, enhancer activity in the ventral diencephalon coincides with another prominent *Wnt*7b expression domain (Lee et al., 2000; Parr et al., 1993). Alternatively, control of *Emx*2 expression could involve other yet to be identified Wnt genes with expression in the cortical neuroepithelium. The Tcf-binding site alone, however, only confers weak *lac*Z expression in the telencephalon, suggesting a requirement for additional factors (see later). Although *Bmp* expression and signalling is mainly confined to the dorsomedial telencephalon, our mutational analysis suggests an important role for the Smad-binding site in this regulation.

While our data establish Bmps and Wnts as essential components of the molecular mechanisms governing regional *Emx*2 expression, several lines of evidence suggest that activation of Bmp and Wnt signalling is not sufficient for the induction of *Emx*2 expression during normal development. First, even within the neural tube, co-expression of several Wnt and Bmp genes occurs more widespread (Furuta et al., 1997; Parr et al., 1993) while *Emx*2 transcription as well as *Emx*2 enhancer activity are confined to the forebrain. Second, we were able to define a second regulatory element, DT2, which is required for reporter gene expression in the dorsal telencephalon. While DT1 on its own was not sufficient to mediate this activity, a fusion construct consisting of just DT1 and DT2 drove *lac*Z expression in a pattern indistinguishable from the original enhancer construct. This data indicates that DT2 does not solely act to inhibit potential repressive elements within the *Emx*2 enhancer but functions as a positive regulator and synergises with DT1 in the tissue-specific regulation of *Emx*2. Region specific expression of the yet unknown factor(s) binding to the DT2 element might therefore be responsible for conferring forebrain specific activation of the *Emx*2 enhancer.

A genetic hierarchy controlling dorsal telencephalic development

Our findings on the regulation of *Emx*2 expression begin to unravel a genetic hierarchy controlling development of the dorsal telencephalon. Based upon the severity of the Xt^J phenotype, *Gli*3 constitutes a major regulator of telencephalic development. Loss of Bmp and Wnt gene expression in this mutant may be explained by a direct interaction between Gli3 and the promoters of these genes which is supported by findings in *Drosophila* parasegment and wing imaginal disc development, where Ci activates *wingless* and *dpp* expression, respectively (Von Ohlen and Hooper, 1997; Hepker et al., 1999). Similarly, Wnt genes have recently been identified as targets and mediators of Gli function during vertebrate development (Mullor et al., 2001). However, the genetic studies on forebrain development in Xt^J mice do not allow us to distinguish between a direct/indirect regulation. Furthermore, it remains to be clarified, whether Bmps and Wnts might independently be regulated by Gli3. Wnt proteins might be required to induce Bmp expression in the dorsomedial telencephalon or vice versa. An analysis of the regulatory elements directing Wnt and Bmp gene expression in the dorsal telencephalon will be required to distinguish between these possibilities.

The identification of Wnts/Bmps as regulators of *Emx*2 expression places this homeobox gene downstream of these signalling pathways in the genetic hierarchy controlling

telencephalic development. Consistent with this idea, hippocampal development is affected by both the *Wnt*3a and the *Emx*2 mutation though to different extents. Similar to the *Gli*3 mutation, loss of *Wnt*3a function leads to a loss of the hippocampus (Lee et al., 2000), while it is reduced in size in the *Emx*2 mutant (Pellegrini et al., 1996; Tole et al., 2000a; Yoshida et al., 1997). This difference suggests the involvement of Wnt target genes other than *Emx*2 in the control of this developmental process such as the *Lhx*5 homeobox gene (Zhao et al., 1999). In addition, a role for Bmps in *Emx*2 regulation could be demonstrated by the finding that ectopic expression of *Bmp4* throughout the dorsal telencephalon as observed in *Bf1* mutant mice coincides with an expansion of the *Emx*2 expression domain into the ventral telencephalon (Dou et al., 1999; Xuan et al., 1995). Furthermore, the unaltered expression patterns of *Gli*3 and *Wnt* genes in the *Emx*2 mutant telencephalon (Yoshida et al., 1997) (data not shown) show that these genes are not regulated by *Emx*2.

Relationship between *Emx2* regulation and function

During its development the mammalian neocortex acquires different regional characteristics that anticipate its functional specification in different cortical areas. The molecular mechanisms leading to areal specification and differentiation are largely unknown but recent studies have implicated the *Pax*6 and *Emx*2 homeobox genes in the acquisition of regional identity in the cerebral cortex (Bishop et al., 2000; Mallamaci et al., 2000b). Loss-of-function mutations of either gene lead to an expansion of certain cortical domains at the expense of other areas. This role appears to be intimately linked to opposing gradients of *Pax*6 and *Emx*2 expression (Bishop et al., 2000) A better understanding of the roles of these homeobox genes in regulating neocortical arealisation requires the characterisation of the patterning mechanisms that govern their graded expression. In this regard, our findings on the cooperative interaction of Wnt and Bmp signalling in the control of *Emx*2 expression represent a first step in the elucidation of these mechanisms as the confinement of this synergy to the dorsomedial telencephalon ensures the highest expression level in this tissue and helps to establish an *Emx*2 expression gradient along the mediolateral axis of the telencephalon. The identification of *Emx*2 as a direct target of Wnt/Bmp signalling therefore provides an important insight into the molecular control of neocortical arealisation.

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