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Dynamic Domains of Gene Expression in the Early Avian Forebrain

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The expression domains of genes implicated in forebrain patterning often share borders at specific anteroposterior positions. This observation lies at the heart of the prosomeric model, which proposes that such shared borders coincide with proposed compartment boundaries and that specific combinations of genes expressed within each compartment are responsible for its patterning. Thus, genes such as *Emx1*, *Emx2*, *Pax6*, and *qin* (*Bf1*) are seen as being responsible for specifying different regions in the forebrain (diencephalon and telencephalon). However, the early expression of these genes, before the appearance of putative compartment boundaries, has not been characterized. In order to determine whether they have stable expression domains before this stage, we have compared mRNA expression of each of the above genes, relative both to one another and to morphological landmarks, in closely staged chick embryos. We find that, between HH stage 8 and HH stage 13, each of the genes has a dynamic spatial and temporal expression pattern. To test for autonomy of gene expression in the prosencephalon, we grafted tissue from this region to more caudal positions in the neural tube and analyzed for expression of *Emx1*, *Emx2*, *qin*, or *Pax6*. We find that gene expression is autonomous in prosencephalic tissue from as early as HH stage 8. In the case of *Emx1*, our data suggest that, from as early stage 8, presumptive telencephalic tissue also is committed to express this gene. We propose that early patterning along the anteroposterior axis of the presumptive telencephalon occurs across a field that is subdivided by different combinations of genes, with some overlapping areas, but without either sharp boundaries or stable interfaces between expression domains.

Key Words: **forebrain; gene expression; *Emx1*; *Emx2*; *qin*; *Pax6*; prosomeres; chick embryo.**

INTRODUCTION

The adult forebrain is composed of a variety of discrete regions characterized by diverse neuronal morphology and connectivity. A number of regulatory genes have been implicated in the mechanisms underlying regional specification during early stages of forebrain development. Regionally restricted gene-expression patterns and an apparent division of the prosencephalon into developmental compartments have contributed to the recent emergence of segmental (prosomeric) models of forebrain development (Figdor and Stern, 1993; Rubenstein *et al.*, 1994). Prosomeres have been described as lineage-restricted compartments with conserved boundaries and discrete gene expression (Puelles and Rubenstein, 1993). Thus far, the only evidence that the forebrain is subdivided into lineage-restricted compartments is for the diencephalon, where Figdor and Stern (1993) have shown that dye-labeled cells respect transverse morphological boundaries. However, Golden *et al.* (1996, 1997), using a retroviral cell-marking approach, found that early-marked clones could span the entire anteroposterior extent of the diencephalon, suggesting that restrictions, if they exist, must be transient. Similar cell-marking data show that the telencephalon also lacks a compartmental organization (Szele and Cepko, 1998). Thus, there are conflicting views as to whether the forebrain is truly segmented, with developmental compartments forming the fundamental units of patterning. An additional and perhaps alternative mechanism for patterning the early telencephalon would involve planar signaling from the anterior ectoderm at the pole of the neural plate during gastrulation (Shimamura and Rubenstein, 1997; Houart *et al.*, 1998; Martinez-Barbera *et al.*,

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A

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GTGTCCCAGG AGAGCCTCCT CCTTCACGGC CCCTTCGCCC GCAAACCCAA ACGTATCCGC ACCGCCTTCT CCCCA     75
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TCCCAGCTGC TACGGTTGGA AAGGGCCTTC GAGAAGAACC ATTACGTGGT GGGCGCCGAG CGCAAGCAGC TGGCC    150
SerGlnLeuL euArgLeuGl uArgAlaPhe GluLysAsnH isTyrValVa lGlyAlaGlu ArgLysGlnL euAla

AGCAGCCTCA GCCTCTCCGA GACCCAGGTG AAAGTGTGGT TCCAGAACCG GCGGACGAAA TACAAACGGC AGAAG    225
SerSerLeuS erLeuSerGl uThrGlnVal LysValTrpP heGlnAsnAr gArgThrLys TyrLysArgG lnLys

CTGGAGGAGG AAGGGCCGGA CTCGGAGCAG AAGAAGAAAG GTTCCCATCA CATCAACCGA TGGCGCCTCG CCACC    300
LeuGluGluG luGlyProAs pSerGluGln LysLysLysG lySerHisHi sIleAsnArg TrpArgLeuA laThr

AAGCAGTCGA GCGGAGAAGA CATCGACGTC ACCTCCAACG ACTAA                                    345
LysGlnSerS erGlyGluAs pIleAspVal ThrSerAsnA spStop
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B

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GCGGGGCACC GCGTTACACC CAGTCACTCC CCGCTGCAAA CTTCCCCGGA TCGCCTCCTC CCGCGCACCG CAGAG     75
CCACGCACAG ATTTGCCGGG GGACGCCCGC CGCCGCCGCA CCATGTTTCA GCCCGCGCCC AAGCGCTGTT TCACC    150
                                          MetPheGl nProAlaPro LysArgCysP heThr

ATCGAGTCGC TGGTGGCCAA AGACAGCCCC TTGCCCGCGT CTCGCTCCGA GGATCCCATC CGCCCGGCGG CTCTC    225
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CCGGACTTGG TCTTTGCGGA RGCCGTCTCC CACCCGCCGA ACCCGGCCGT GCCCGTGCAC CCCGTGCCCC CTCCC    375
ProAspLeuV alPheAlaGl uAlaValSer HisProProA snProAlaVa lProValHis ProValProP roPro

CACGCCCTGG CCGCCCATCC GCTGCCCGCC TCGCACTCCA CGCACCCGCT CTTCGCTTCG CAGCAAAGGG ATCCC    450
HisAlaLeuA laAlaHisPr oLeuProAla SerHisSerT hrHisProLe uPheAlaSer GlnGlnArgA spPro

TCCACCTTCT ACCCGTGGCT AATACACCGC TACCGGTATC TGGGCCACAG GTTCCAAGGG AACGAGACCA GCCCG    525
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CTGAGACTGG AACATGCCTT TGAGAAGAAC CATTATGTGG TGGGAGCGGA GAGAAAGCAG CTGGCACACA GCCTC    675
LeuArgLeuG luHisAlaPh eGluLysAsn HisTyrValV alGlyAlaGl uArgLysGln LeuAlaHisS erLeu

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AGTCCAGAAG AAATCGACGT CACGTCGGAC GATTAAAAAA CTGGCACTTT GGGACTTTTC AAGCCAGCCA ACGCA    900
SerProGluG luIleAspVa lThrSerAsp AspStop
TCGAGAGAAA GTGTTAAAGG CTCCCTCACC CCGGCGTGGC GGTGCGGGGA AGGGACACGG AGAGCTTCAT CCAAA    975
CGCGATTCCC GTCTGGAGGG CGGCGGCGAA CGGCGGGGGC AGAGCGCGAG GTGCGGAGCG CGGCGCTGCC GGCGC   1050
GGCCCTGGCA CGGGGATCTG CCGGGCAGAG CCCAGCGCCG GGACGAGGGA ATGACTGACT GACTGACTGA CTGAC   1125
AGGTATTCCG TTTCTCGCAG TCCACTTTTA AAA                                                 1158
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FIG. 1. Sequence analysis of c*Emx1* and c*Emx2*. (A) Nucleotide sequence of chick *Emx1* cDNA. This sequence contains only partial coding, but does include the homeobox and the 5′ end of the coding region, including the stop site. (B) Nucleotide sequence of chick *Emx2* cDNA. The clone contains the entire coding region, including 100 bp of 5′ UTR and approximately 300 bp of 3′ UTR. The 3′ UTR was used in the *in situ* probe. The entire coding region of this gene is 741 bp. (C, D) Amino acid comparisons of chick, human, *Xenopus*, and zebrafish *EMX1* and human, chick, and mouse *EMX2*, respectively. Arrows represent the homeobox. (E) Comparison of both family members of *cEMX*. Arrows represent the homeobox.

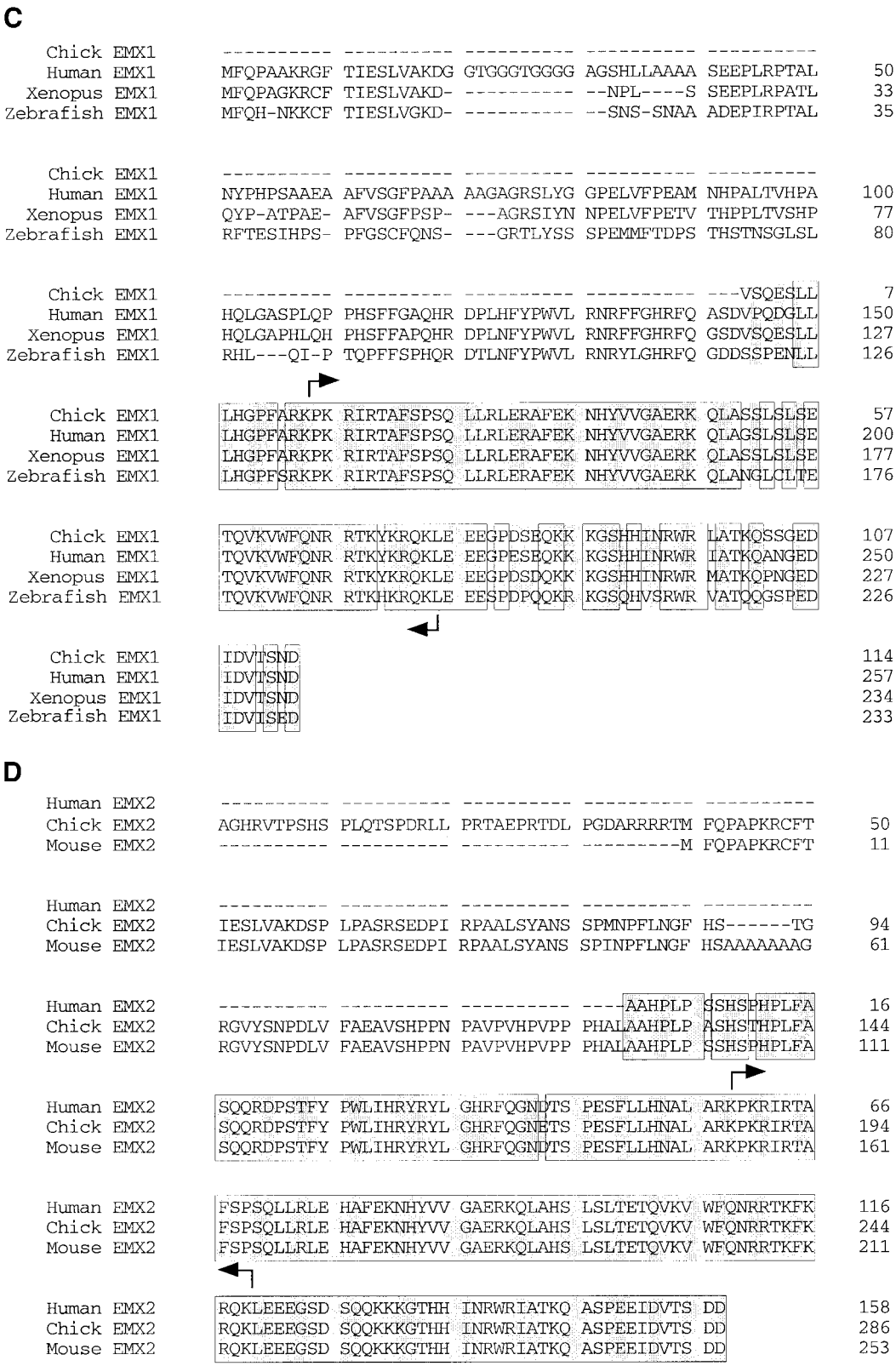


FIG. 1—*Continued*

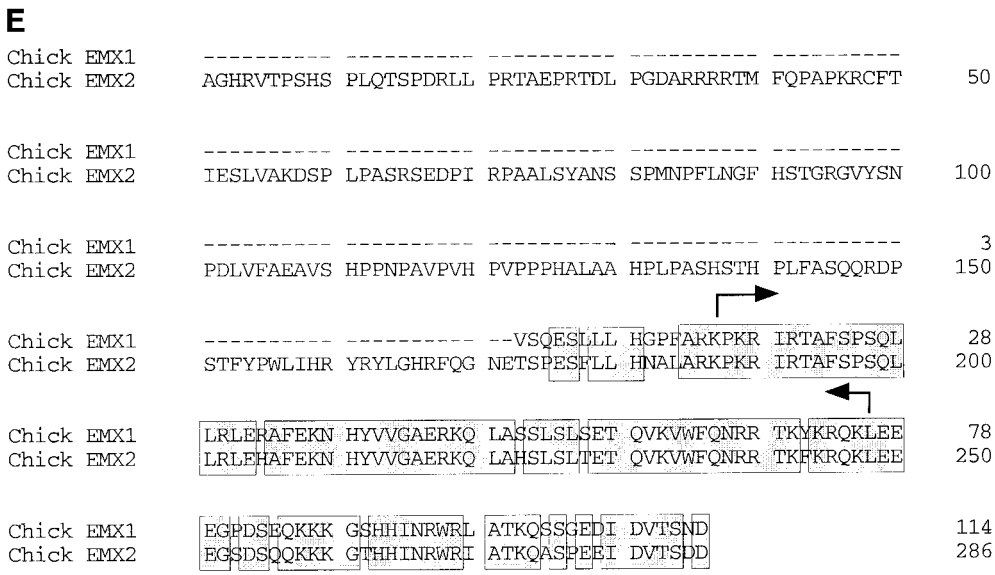


FIG. 1—*Continued*

2000) and/or vertical signaling from the underlying endomesoderm (Jones *et al.*, 1999; Ho *et al.*, 1999).

In contrast to the forebrain, there are compelling data that the hindbrain is a segmented region of the neuraxis (Lumsden and Krumlauf, 1996). Hindbrain segment (rhombomere) boundaries form between HH (Hamburger and Hamilton, 1951) stages 9–12. The expression of many hindbrain genes, for example *Krox20* (Irving *et al.*, 1996) and *Hoxa2* (Prince and Lumsden, 1994), is initiated before the presence of morphological boundaries, yet their expression borders are conserved and stable with respect to each other from the onset of their expression (Lumsden and Krumlauf, 1996). Are the early patterning mechanisms in the prosencephalon, before the appearance of putative compartments, analogous to those of the hindbrain?

The approach we have taken to examine this issue has been to analyze regional expression of several genes during early stages of forebrain development, focusing on the spatial and temporal expression patterns of putative determinants of regional character: *Emx1, Emx2, qin,* and *Pax6*. The expression patterns of *Emx1* and *Emx2* have previously been described during development (Simeone *et al.*, 1992a,b; Morita *et al.*, 1995; Gulisano *et al.*, 1996; Fernandez *et al.*, 1998; Pannese *et al.*, 1998). Transcripts of these two genes are restricted to the dorsal aspect of the telencephalon. *Emx2* transcripts are also seen in the ventral diencephalon. Targeted null mutation of the *Emx2* gene has revealed selective loss of regions, in particular the dentate gyrus (Yoshida *et al.*, 1997; Pellegrini *et al.*, 1997; Tole *et al.*, 2000). In contrast, only subtle defects were detected in *Emx1*-null mutants (Yoshida *et al.*, 1997). That a more severe phenotype was not detected could be due to the fact that, at early stages, *Emx1* is expressed within the domain of *Emx2*, and partial functional redundancy could exist between these family members. Recent evidence suggests that *Emx2* functions downstream of Gli3, a negative regulator of *Shh* (Theil *et al.*, 1999). *Qin* (Tao and Lai, 1992; Hatini *et al.*, 1994; Tole and Patterson, 1995; Chang *et al.*, 1995) is expressed throughout the telencephalon, and *Pax6* (Goulding *et al.*, 1993; Li *et al.*, 1994) is expressed in the dorsal telencephalon as well as the diencephalon. Targeted null mutation of *Bf1* (the murine homologue of *qin*) results in the loss of the ventral telencephalon and a reduced dorsal region (Xuan *et al.*, 1995). Small-eye mice, which carry mutations in the *Pax6* gene, also have forebrain abnormalities (Stoykova *et al.*, 1996; Warren and Price, 1997).

We looked at the relationship between the early expression of these genes to see whether there are stable expression boundaries during HH stages 8–13. If, as has been suggested, the forebrain develops in a similar manner to the hindbrain, the expression patterns of these genes would be expected to be stable with respect to each other. However, we found that the genes have dynamic expression patterns and that the boundaries of expression change in relation to each other during these early stages.

It has previously been demonstrated that, when cells are transplanted singly from one region to another, either between the dorsal and ventral telencephalon or to different positions along the anterior–posterior axis, they adopt the phenotype of their new neighbors (Fishell, 1995; Brustle *et al.*, 1995), suggesting that local cues in the new environment can influence solitary cells and change their identity. This has more recently been investigated at the molecular level: for example, BF1-positive cells maintain their molecular identity after integrating into regions where they are not normally found (Na *et al.*, 1998). This implies that, even

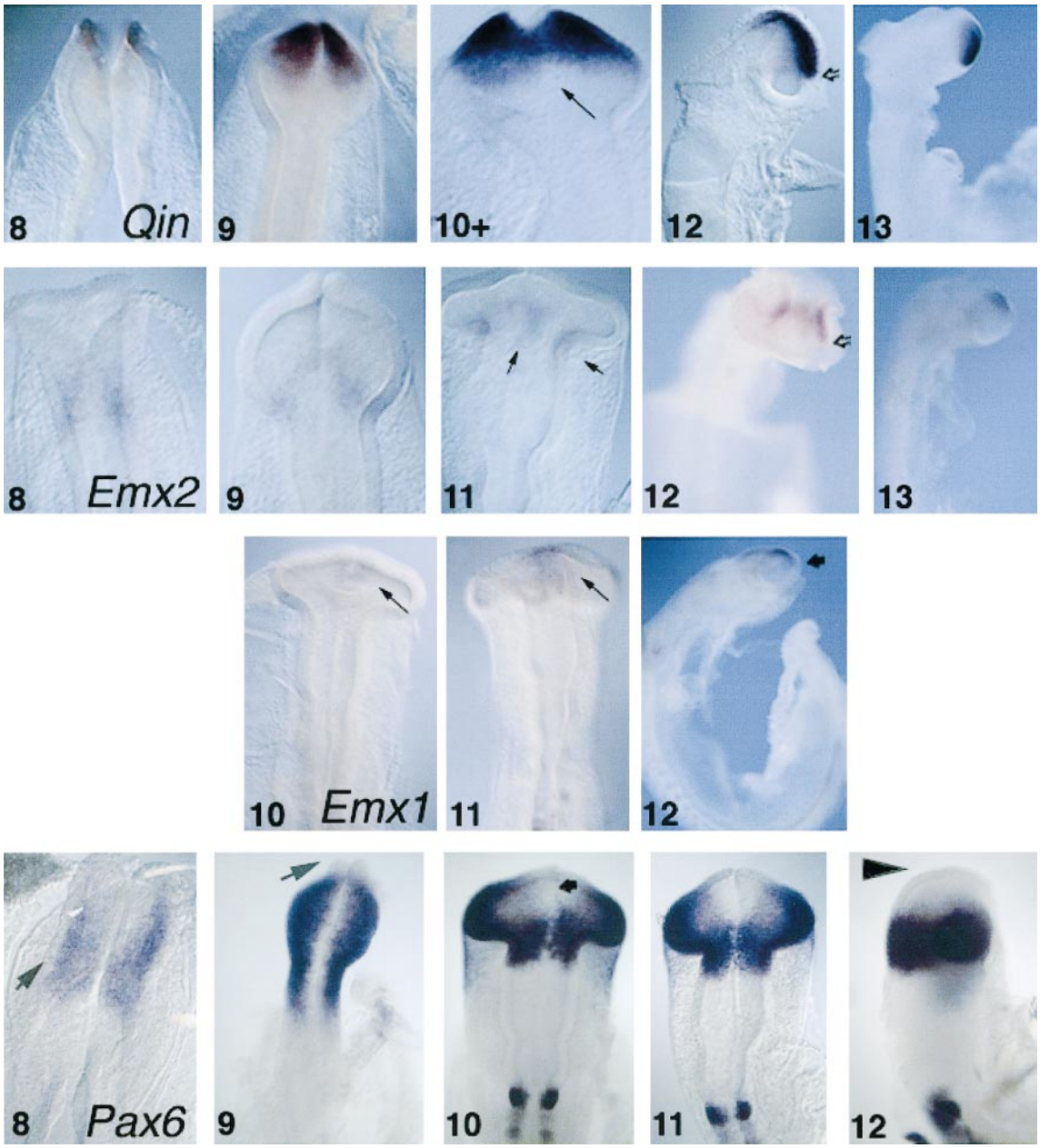


FIG. 2. Expression patterns of *qin*, *Emx1*, *Emx2*, and *Pax6* along the A-P axis of the prosencephalon during early development. Top row: *qin*. At HH8, mRNA transcripts are detected at the anterior end of the neural folds and gradually extend into caudal regions as the prosencephalon develops. At HH10+, *qin* is detected in the rostral half of the prosencephalon (see filled arrow). By HH12–13, *qin* is detected mainly in the ventral part of the telencephalon (open arrow denotes ventral extent). Second row: *Emx2*. At HH8 and HH9, two stripes are detected in the prosencephalon. By HH11, expression has progressed anteriorly and fills the core of the prosencephalon (filled arrows). By HH12–13, expression is restricted to the dorsal telencephalon (open arrow). Third row: *Emx1*. At HH10, *Emx1* is restricted to the anterior end of the prosencephalon (filled arrow). By HH11/12, *Emx1* is expressed in the dorsal telencephalon (open arrow). Bottom row: *Pax6*. At HH8, *Pax6* is expressed in the caudal half of the prosencephalon (filled arrow), with lateral expression in the anterior half. At HH9, *Pax6* transcripts are detected throughout the prosencephalon, except for the most anterior region (filled arrow) and midline. By HH11–12, *Pax6* continues to be expressed in the prosencephalon, though not anteriorly (open arrow). At HH12, *Pax6* expression remains restricted to the diencephalon and absent from the telencephalon (large open arrow). HH8–11, dorsal view; HH12–13, lateral view.

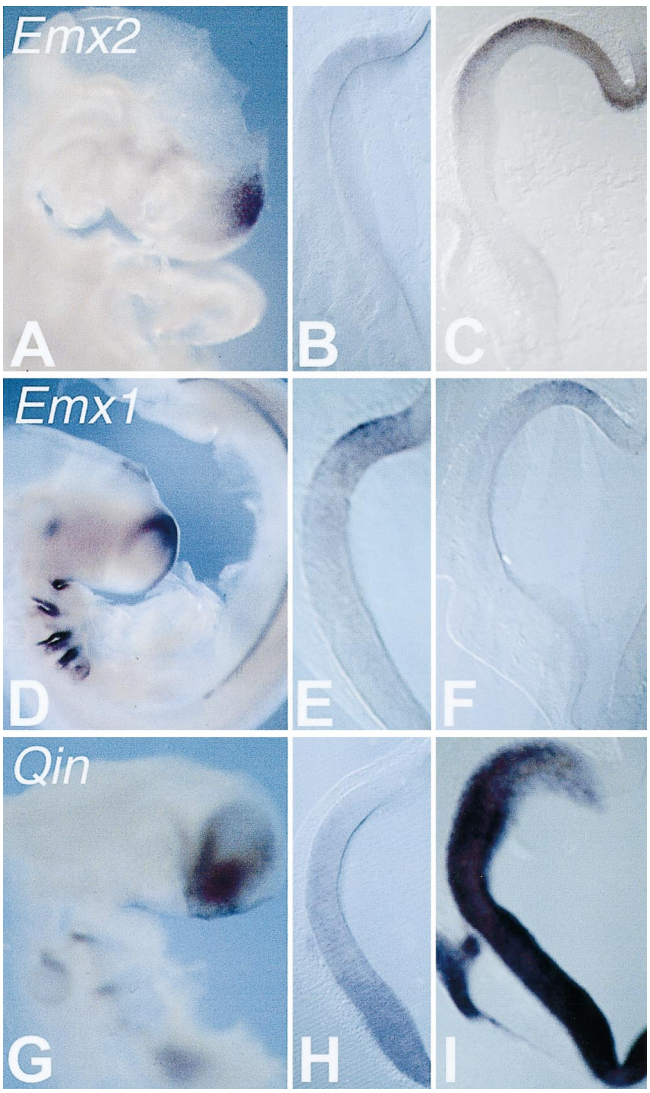


FIG. 3. Expression of *Emx2*, *Emx1*, and *qin* at HH17 and HH19. (A–C) *Emx2*; (D–F) *Emx1*; (G–I) *qin*. (A, D, G) Whole-mount *in situ* hybridizations of HH17 chick embryos. (B, E, H) Coronal sections through the telencephalon of HH17 embryos. (C, F, I) Coronal sections through the telencephalon of HH19 embryos. All are dorsal to the top.

though these cells appear morphologically different, their molecular identity does not change. Indeed, fate-map studies (Couly and LeDouarin, 1987, 1988; Fernandez *et al.*, 1998) suggest that regions have a determined fate from early stages. Previous experiments have also investigated the commitment of prosencephalon to a forebrain fate from early stages and find that caudal forebrain is competent to express *En2* when grafted near the *En2*-polarising region (Bloch-Gallego *et al.*, 1996). However, the same study demonstrated that more anterior tissue (rostral diencephalon and telencephalon) does not change fate (as assessed by the lack of *En2* induction) when grafted heterotopically. Conversely, it has also been shown that transplantation of prosencephalon into the mesencephalon results in the down-regulation of *Pax6* expression after 72 h (Nomura *et al.*, 1998). Thus, we were interested in the commitment of rostral prosencephalon to express early telencephalic markers (*Emx1*, *Emx2*, *qin*, and *Pax6*) when transplanted more posteriorly within the embryo. We find that heterotopic transplants of telencephalic tissue exhibit autonomy of gene expression from as early as HH stage 8, before compartmental boundaries are supposed to become established (Figdor and Stern, 1993).

MATERIALS AND METHODS

Cloning of cEmx1 and cEmx2

A cDNA library in Lambda ZAPII (Stratagene, La Jolla), prepared from HH14–17 chick embryos, was screened at low stringency with a mouse *Emx1* genomic sequence corresponding to the third exon (Simeone *et al.*, 1992). We followed the automatic excision protocol, following the kit instructions. Two primers were designed for the *Emx2* homeobox and used to amplify a 175-bp fragment of *Emx2* from chicken cDNA. The primers used were: 3′-GGggatccAAGCGIAT(C/T)CG(C/I)AC(C/I)GC(C/I)TT and 5′-CTaagctt(C/T)TGIC(T/G)(C/T)TT(A/G)(A/T)A-(C/T)TTIGT. The cloned PCR fragment was used to screen a HH12–16 cDNA library in Lambda ZAPII, which was a kind gift from Dr. David Wilkinson. Hybridization was carried out in 50% formamide overnight at 42°C. A 1.2-kb clone was obtained and subcloned into pKS-bluescript. The product was sequenced by using dye-terminator chemistry with an Amplitaq FS DNA sequencing kit and analyzed with a ABI 377 Prism DNA sequencer (ABI/Perkin-Elmer) revealing 700 bp (base pair) of full coding.

Preparation of Riboprobes

Digoxigenin-labeled (DIG) riboprobes were used to detect mRNA transcripts. For the *Emx1* probe, a fragment of 260 bp was subcloned into pGEM3 (Promega, U.K.). This included the third helix of the homeobox to the end of the clone. The *Emx2* probe was prepared by digesting the full-length clone with *Pvu*II and transcribing with T7 (Promega). This generated a 500-bp RNA probe containing mainly 3′UTR. The *qin*, *Pax6*, and *Dlx2* probes have been described previously (Chang *et al.*, 1995; Goulding *et al.*, 1993; Begbie *et al.*, 1999).

Whole-Mount RNA in Situ Hybridization

Whole-mount RNA *in situ* hybridization was according to the published protocols of Wilkinson (1992) and Henriqué *et al.* (1995). Embryos to be sectioned were embedded in 20% gelatin (in PBS) and refixed in 4% PFA overnight at 4°C. Sections were cut on a vibratome at 50 or 100 μm and mounted in 90% glycerol/PBS.

Grafting and Immunohistochemistry

Hens eggs were supplied by Poydon Farm, Hertfordshire, and incubated at 38°C. Donor tissue was prepared from HH8–18 embryos. Donor embryos were removed from the egg and incubated

in a solution of dispase (1 mg/ml in L15) for 10 min (HH8 and HH9) or 20 min (HH10 and older) at room temperature. They were extensively washed in Ringer's to remove the dispase and left in Howard's Ringer containing 10 μg/ml DNase. Pieces of the anterior or posterior prosencephalon, and dorsal or ventral telencephalon were carefully dissected away and grafted heterotopically into the mesencephalon or hindbrain of similar staged host embryos. Operated embryos were sacrificed 48 h later. QCPN whole-mount antibody staining was performed as described by Koentges and Lumsden (1996).

RESULTS

Isolation and Cloning of cEmx1 and cEmx2

We isolated and sequenced cDNAs of c*Emx1* and c*Emx2*. We obtained partial sequence of c*Emx1*, which included the homeobox and up to the 5′ end of the coding region, including the stop site (Fig. 1A) and the full-length coding region of *Emx2*, including some 3′ and 5′ UTR (Fig. 1B). The amino acid sequences of these clones were compared with previously published sequences of their vertebrate homologues. For the five species (Simeone *et al.*, 1992b; Morita *et al.*, 1995) across which we compared *Emx1*, there was 78% amino acid identity (Fig. 1C). Chick *Emx2* and its mouse (Simeone *et al.*, 1992b) counterpart shared 96% amino acid identity throughout the coding region (Fig. 1D). We also compared c*Emx1* and c*Emx2* to see how high the homology was between family members. They had 93% predicted amino acid identity across the homeobox (Fig. 1E).

Early Expression Patterns of Emx1, Emx2, qin, and Pax6 Show Dynamic Boundaries

HH stages 8–9. We detected qin transcripts from as early as HH6 (data not shown). At HH8, strong staining is restricted to the anterior neural plate (Fig. 2). Expression of *Emx2* was initially detected at around HH8 (Fig. 2) as a transverse band across the anterior neural plate. However, at this stage, the expression of *Emx2* is very weak. There is a gap between the *qin* and *Emx2* regions, with *Emx2* being located more posteriorly within the presumptive prosencephalon. We also saw *Pax6* expression at HH8, when it partially overlaps with the ventral–medial expression of *Emx2*. *Pax6* is not detected in the dorsal lateral region of *Emx2* expression (Fig. 2, arrow). The early expression of *qin*, *Emx2*, and *Pax6* is highly dynamic. At HH8, they have similar caudal limits of expression. By HH9, *Emx2* mRNA is detected in two bands, which are found in the posterior half of the prosencephalon (Fig. 2), expression is still relatively weak. In contrast, *qin* encompasses the anterior half of this region. These two expression domains appear to abut rather than overlap at this stage, meeting around the middle of the prosencephalon. *Pax6* at HH9 is detected throughout most of the prosencephalon, but is excluded from the most anterior and dorsal medial regions (Fig. 2, arrow). At this stage, *Pax6* has a more caudal limit of expression than has *Emx2*.

HH stages 10–11. We first detected the prosencephalic expression of *Emx1* at approximately HH10−, in only a few cells at the anterior end of the brain (not shown). One somite stage later, at HH10, *Emx1* transcripts are detected in a slightly larger domain of the anterior prosencephalon, though still weak (Fig. 2, filled arrows). By HH11, expression of *Emx1* is seen throughout the anterior/dorsal prosencephalon (Fig. 2, arrow). At this stage, *Emx2* is expressed in the center of the prosencephalon (Fig. 2, filled arrows), just overlapping with *Emx1* medially. *qin* expression is still anterior, contains the *Emx1*-expressing region, and overlaps with *Emx2* in the medial prosencephalon. By contrast, *Pax6* is expressed throughout the prosencephalon, except in the *Emx1*-expressing region. The *Pax6* and *Emx1* domains appear to abut directly onto each other.

HH stages 12–13. During these stages, distinct telencephalic and diencephalic subdivisions of the prosencephalon become visible morphologically. *Emx1* is initially expressed at the anterior end of the telencephalon and then spreads caudally. As the head of the embryo begins to turn, forming the cephalic flexure, the dorsal telencephalon (pallium) is positive for *Emx1* transcripts. By HH12 (Fig. 2, open arrow), *Emx2* is also restricted to the dorsal telencephalon, seen even more clearly by HH13. The expression of *Emx2* differs from *Emx1* in that it is initially expressed in the posterior prosencephalon and spreads to more anterior regions. Like *Emx1*, *Emx2* is expressed in the dorsal telencephalon by HH12–13, but differs in that it is also expressed in the ventral diencephalon (data not shown). By this stage, the expression of *qin* is becoming restricted to the anterior ventral telencephalon, but weak dorsal telencephalic expression remains (open arrow). *Pax6* at HH stages 12–13 is restricted to the diencephalon and is not expressed in the telencephalon until later stages.

It would therefore seem that the early telencephalon is characterized by overlapping and changing domains of expression of these genes. The boundaries of expression become fixed relative to each other only by approximately HH12/13 for *Emx1*, *Emx2* (dorsal telencephalon), and *qin* (ventral and part of dorsal telencephalon) (Fig. 2). The *Pax6* domain is not fixed at this stage but is restricted to the diencephalon (Fig. 2, large arrow).

HH stages 15–20. By HH15–16, *Pax6* is expressed in the dorsal telencephalon (data not shown). By HH17, all four genes discussed here have apparently fixed boundaries of expression. *Emx1*, *Emx2*, and *Pax6* are expressed in the dorsal telencephalon and *qin* is expressed throughout the telencephalon, except in the dorsal-most aspect (Fig. 3). These expression patterns persist through HH20.

We also examined the expression patterns of *Emx1*, *Emx2*, and *Pax6* (markers of the dorsal pallium) in relation to the onset and early expression of *Dlx2* in the ventral telencephalon (subpallium). *Dlx2* is used as a marker of the subpallium (Puelles *et al.*, 1999), although it is also expressed in other regions of the avian brain. The analysis of

expression of both *Emx* genes in conjunction with *Dlx2* reveals a gap between the two expression domains (Figs. 4A and 4B, see *). This gap has already been noted for later developmental stages (Fernandez *et al.*, 1998; Puelles *et al.*, 2000) and has been suggested to correspond to the anlage of the dorsal ventricular ridge (Fernandez *et al.*, 1998). We found that this *Emx*-/*Dlx*-gap is present from as early as stage 20 and that *Pax6* is expressed within it (Figs. 4C and 4D).

In summary, *qin* and *Emx2* are expressed in distinct regions of the prosencephalon at HH8, with *qin* anterior and *Emx2* posterior. *Pax6* at this stage overlaps with *Emx2*. At HH9, *qin* and *Emx2* expression domains abut, but *Pax6* overlaps with both. By HH11, all four genes overlap in certain parts of the prosencephalon. At later stages (HH20), all the genes analyzed (*Emx1*, *Emx2*, *Pax6*, *qin*, and *Dlx2*) respect very specific regions: *Emx1* and *Emx2*, dorsal telencephalon (pallium); *Pax6*, dorsal and intermediate telencephalon (pallium and dorsal ventricular ridge); *qin*, most of the telencephalon, except the most anterior region; *Dlx2*, ventral telencephalon (subpallium).

Different Areas of the Early Forebrain Show Regional Autonomy in Gene Expression

The highly dynamic spatiotemporal expression of the genes analyzed here, especially *Emx1* and *Emx2*, raised the question as to whether telencephalic tissue shows autonomy of gene expression when developing ectopically, and, if so, from what developmental stage. Determination of gene expression was tested at stages when the genes in question are already expressed (see Fig. 2) by grafting small pieces of chick or quail forebrain tissue in place of mesencephalic tissue of stages 9–14 host chick embryos (Fig. 5). We transplanted dorsal telencephalon at HH11–18 to analyze transcripts of both *Emx1* and *Emx2*, posterior prosencephalon at HH8–10 to analyze transcripts of *Emx2* and *Pax6*, and either anterior prosencephalon at HH7–10 or ventral telencephalon to analyze *qin* transcripts. We found in all cases that *Emx1*, *Emx2*, *qin*, and *Pax6* expression was maintained within the grafted tissue, showing that these three genes show regional autonomy after 48 h (Figs. 6B–6I; Table 1). In light of previous data suggesting that the expression of *Pax6* in the graft is maintained for 48 h, then down-regulated (Nomura *et al.*, 1998), we also looked at the expression of *Pax6* after 72 h. In contrast to this earlier report, we found that the ectopic expression of *Pax6* is maintained and does not switch off in the transplanted tissue (Fig. 6H). Indeed, for most of the genes analyzed, the expression within the transplant is similar in intensity to the endogenous expression (Figs. 6F–6H). However, the expression of *Emx1* in the graft was occasionally weaker than the endogenous expression (Fig. 6E). To investigate whether the expression of *Emx1* and *Emx2* would overlap in the transplant, we performed a series of grafts for double (two-color) in situs and found that *Emx1* and *Emx2* colocalized in the graft (Fig. 6I).

We also transplanted tissue from the presumptive dorsal telencephalon to the mesencephalon at stages before the onset of *Emx1* expression (as determined for stage-matched donor embryos by whole-mount *in situ* hybridization), having located the presumptive region by the expression of *Emx2* at HH8 and HH9. We found that *Emx1* still switched on in the transplant (Figs. 6A and 6E; Table 1). This result shows that the fate of the presumptive *Emx1*-expressing tissue is determined with respect to the expression of this gene and no longer labile to the influence of local cues.

DISCUSSION

We have analyzed the early prosencephalic expression of the regulatory genes, *Emx1*, *Emx2*, *qin*, and *Pax6*, to see whether they have fixed boundaries of expression in relation to each other from their onset, as has been demonstrated for regulatory genes during hindbrain development. Expression domains were mapped from HH8, long before putative prosomere boundaries are established. We find that these genes have dynamic spatial and temporal expression patterns (see schematic Fig. 7A). Despite the borders of expression domains changing during HH8–13, the genes are expressed in subregions destined to become specific structures from as early as HH 8/9 (Fig. 7B) according to the fate maps of Couly and LeDouarin (1987, 1988) and Fernandez *et al.* (1998). Thus, *qin* is expressed in the anterior neural plate, which gives rise to the olfactory placode, ectoderm of nasal cavities, and the floor of the telencephalon, all of which express *qin* at later stages (Fig. 3). At HH8, *Emx2* is expressed in the tissue fated to give rise to the neurohypophysis and the roof of the telencephalon, later extending into the dorsal telencephalon and ventral diencephalon. As for *qin*, the early and late expression of *Emx2* matches the fate map. The third gene we examined at HH8 was *Pax6*, expression of which corresponds to the presumptive roof of the telencephalon and diencephalon. *Pax6* expression remains restricted to the diencephalon until approximately HH15 (data not shown), when transcripts become detectable also in the dorsal telencephalon. As shown by Fig. 7A, the expression domains of *qin* and *Emx2* do not overlap at HH8, yet, by HH9, the two domains abut and *Pax6* transcripts now overlap with both *qin* and *Emx2*. At this stage, the fate maps suggest that the expression of *qin* corresponds to the future striatum and the expression of both *Emx2* and *Pax6* to the pallium. By HH10/11, *Pax6* encompasses the entire prosencephalon, except for the anterior-most region, where *Emx1* is expressed.

The early regional expression of *Emx1*, *Emx2*, *qin*, and *Pax6* mark domains that lack clear fixed boundaries, such as would be expected of developmental compartments. The changing extent of overlap of these expression domains implies that such boundaries are either not established at HH8–11 or not respected by these genes. By HH14, how-

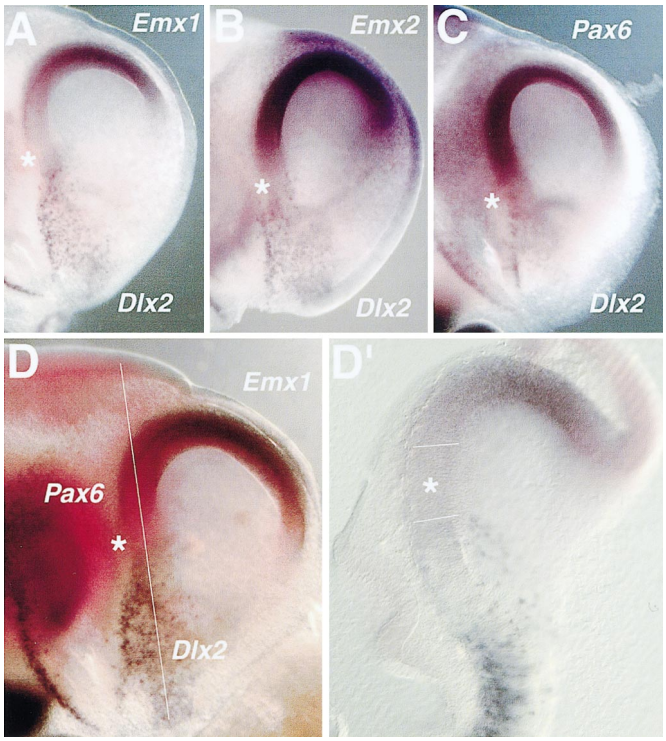


FIG. 4. Expression of dorsal-expressed genes, *Emx1*, *Emx2*, *Pax6* in relation to a ventral-expressed gene, *Dlx2*. (A–C) Double DIG whole-mount *in situ* hybridizations for *Emx1* and *Dlx2* (A), *Emx2* and *Dlx2* (B), and *Pax6* and *Dlx2* (C). The presence or absence of a gap between the expression domains is indicated with an asterisk. (D) Triple whole-mount *in situ* hybridization for *Emx1* and *Dlx2* (blue) and *Pax6* (red). (D′) A 100-μm coronal section through the telencephalon. All views are dorsal to the top and anterior to the right.

ever, sharp boundaries of expression are established for *Emx1*, *Emx2*, and *qin*, while those for *Pax6* do not become stable until HH16. By HH20, all the genes described here demarcate specific regions of the telencephalon, as demonstrated by the comparison of the expression of these genes with that of *Dlx2*.

These data imply that telencephalic patterning differs from that of the hindbrain, where the onset of expression of pattern-regulating genes (e.g., *Krox20*, Wilkinson *et al.*, 1989a; *Hoxb* genes, Wilkinson *et al.*, 1989b) more closely matches the establishment of compartment properties (Lumsden and Krumlauf, 1996). The existence of lineage-restricted compartments is not disputed for the hindbrain, but remains controversial for the forebrain (Rubenstein *et al.*, 1994; Figdor and Stern, 1993; Golden and Cepko, 1996; Golden *et al.*, 1997; Szele and Cepko, 1998). The principal support for a compartmental organization of the forebrain rests on gene expression data from comparatively late stages of development (Rubenstein *et al.*, 1994). We show here that the early boundaries of expression of representative forebrain genes are in fact highly dynamic. While these data do not deny the existence of "molecular prosomeres," they do suggest that such modules do not come into force until as late as HH14, when expression boundaries become stabilized.

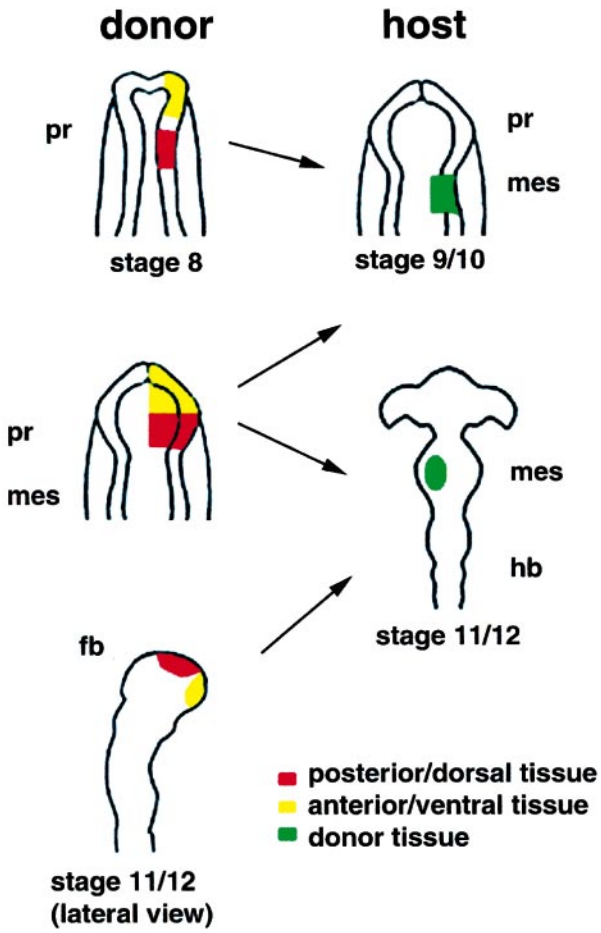
The changing boundaries of early gene expression suggested a simple test to see whether these genes show autonomy from as early as HH8. *Emx1*, *Emx2*, *qin*, and *Pax6* were still expressed in the tissue in its heterotopic location and did not switch off in response to local cues. Furthermore, in contrast to a previous study where *Pax6* was seen to down-regulate in ectopic transplants after 48 h (Nomura *et al.*, 1998), we found that *Pax6* was still strongly expressed in the transplants 72 h after grafting. The fact that all of these genes show autonomy of gene expression within the grafts is in apparent contrast to single cell transplant experiments, which have shown that single cells in a new environment respond to local cues and adopt the morphology of their new neighbors; single cells were not committed in respect of morphology (Fishell, 1995; Brustle *et al.*, 1995). Although molecular expression was not analyzed in these studies, a subsequent analysis of single forebrain cell transplants has demonstrated commitment in respect of *Bf1* (*qin*) expression (Na *et al.*, 1998). Similarly, isthmic tissue maintains its original *engrailed* gene expression when grafted into the prosencephalon (Itasaki *et al.*, 1991). It would be of interest in future experiments to transplant individual telencephalic cells heterotopically and see whether they maintain their gene expression. It is possible that by transplanting groups of cells there is a community effect. However, the results of Na *et al.* (1998) suggest that the cells would maintain their original molecular identity.

Recent studies have demonstrated that the prechordal

TABLE 1

Analysis of Grafts of Anterior/Posterior Prosencephalon or Dorsal/Ventral Telencephalon into the Midbrain of Similar Staged Chick Embryos

Stage of donor	Number analyzed with graft intact (48 h)					72 h
	Emx1	*Emx2*	*qin*	*Emx1/Emx2*	*Pax6*	*Pax6*
7+			2			
8	2					
9	9	9		7	11	3
10		1	2			
11	1	3	1			
12	4	2	2			
13	2	2	3			
14	1	1	1			
16	1					
17	1					
18	1					
Total number	22	18	11	7	11	3



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mesendoderm plays an important role in patterning the anterior neural ectoderm from as early as HH5 (Pera and Kessell, 1997; Foley *et al.*, 1997). Our data show that regional autonomy exists from as early as HH8 in the prosencephalon, by which time the early patterning signals have already been set up. Our data also provide an *in vivo* complement to the *in vitro* data of Nakagawa *et al.* (1996), where neuroepithelial explants from E11.5 rat telencephalon maintained the endogeneous expression of genes, including *Emx2*. We have taken this further by showing that the forebrain is committed to express *Emx1* before its normal onset of expression, as determined by whole-mount *in situ* hybridization. In addition, pieces of prosencephalon grafted before the expression of *Emx1* were also analysed for both *Emx1* and *Emx2* within the same tissue. We find that these two genes are expressed within the graft in an overlapping pattern reminiscent of their normal expression.

We have demonstrated that the expression of the telencephalic markers *Emx1*, *Emx2*, *qin*, and *Pax6* is established and autonomous by HH8, but expression domain boundaries remain highly dynamic for a considerable time later. This is consistent with the idea that the prosencephalon initially develops as a uniform field, before and without the action of putative prosomeric boundaries that may appear at later stages (Figdor and Stern, 1993).

FIG. 5. Summary of approach taken to analyze whether c*Emx1*, *Emx2*, *qin*, and *Pa6* show regional autonomy. Grafts were performed by using donor tissue between HH7 and HH18. Pieces of tissue were removed from the donor embryo (red represents posterior graft origins from HH7–10 and dorsal grafts from HH11–18 embryos; yellow represents anterior HH7–10 and ventral HH11–18). These were grafted in place of a similar sized hole that had been made in the mesencephalon of the host embryo (green). All views are dorsal, except the st11/12 donor; pr, prosencephalon; tel, telencephalon; di, diencephalon; mes, mesencephalon; fb, forebrain; hb, hindbrain.

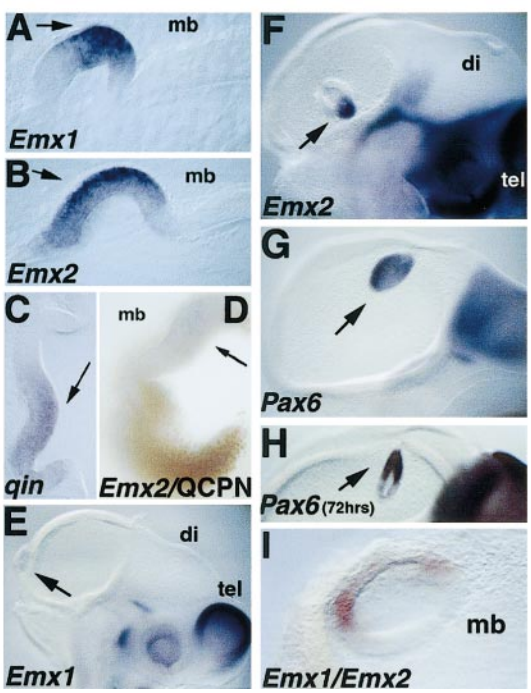

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FIG. 6. Analysis of *Emx1*, *Emx2*, *qin*, and *Pax6* expression in heterotopic grafts. (A–D) After *in situ* hybridization for *Emx1*, *Emx2*, or *qin*, embryos were embedded in gelatin and sectioned at 50 μm. (A) HH9 posterior donor tissue probed for *Emx1*. (B) HH11 dorsal donor tissue probed for *Emx2*. (C) HH12 ventral donor tissue probed for *qin*. (D) HH14 dorsal donor tissue, immunohistochemistry for quail donor cells (QCPN antibody, brown), and *in situ* hybridization for *Emx2* (purple). (E–H) Whole-mount *in situ* hybridization for *Emx1*, *Emx2*, or *Pax6* expression comparing endogenous and ectopic expression. (E) HH9 posterior donor tissue probed for *Emx1*. (F) HH9 posterior donor tissue probed for *Emx2*. (G) HH9 posterior donor tissue probed for *Pax6*. (H) HH9 posterior donor tissue probed for *Pax6* after 72 h. (I) HH9 posterior donor tissue probed for both *Emx1* (blue) and *Emx2* (red). Arrows in (A–H) show the expression of the gene in the transplanted tissue (tel, telencephalon; di, diencephalon; mb, midbrain; hb, hindbrain).

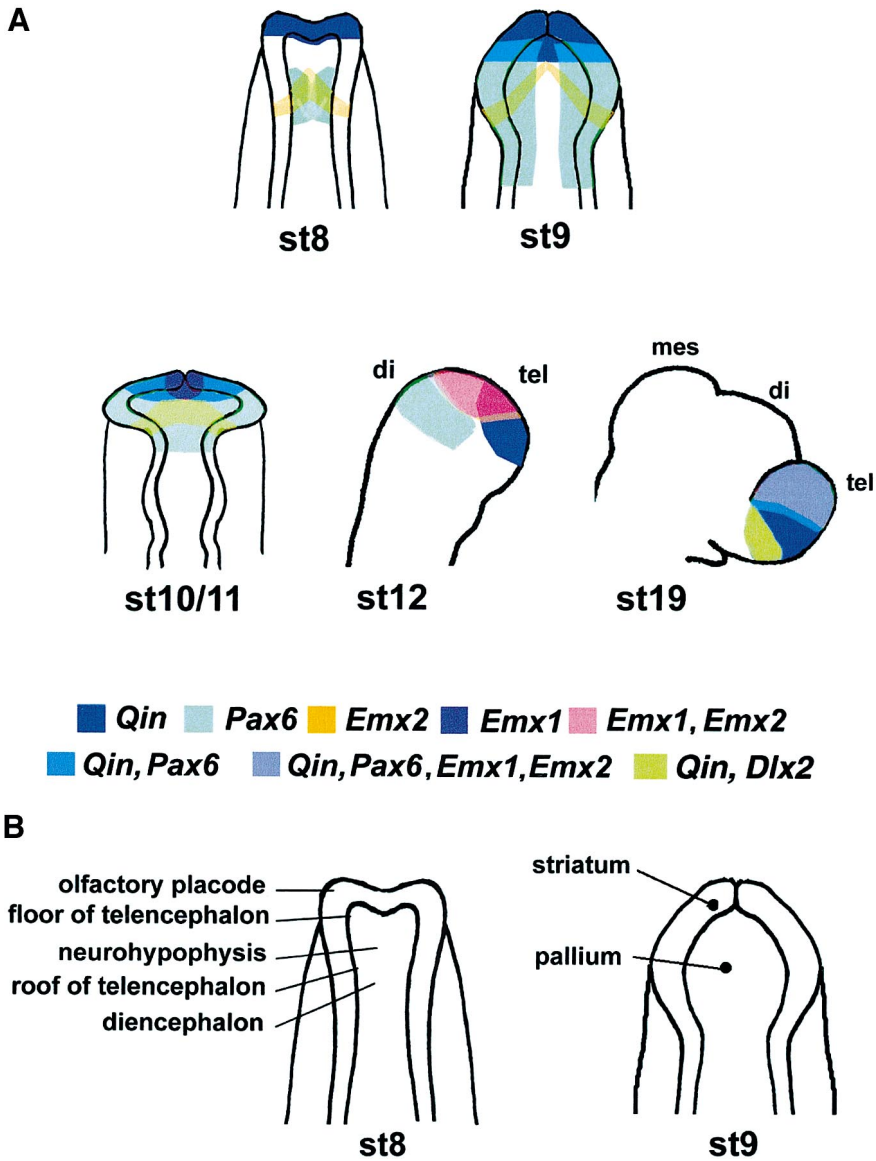

FIG. 7. *Emx1*, *Emx2*, *Pax6*, and *qin* have dynamic spatial and temporal expression patterns during early development of the forebrain. (A) Schematic of the distribution of transcripts along the A-P axis of the forebrain between HH8 and HH19. (B) Summary fate maps of HH8 and HH9 forebrains, showing presumptive forebrain regions (Couly and LeDouarin, 1987; Fernandez *et al.*, 1998).

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