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Two vertebrate homeobox genes related to the *Drosophila empty spiracles* gene are expressed in the embryonic cerebral cortex

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We cloned two homeobox genes, *Emx1* and *Emx2*, related to *empty spiracles*, a gene expressed in very anterior body regions during early *Drosophila* embryogenesis, and studied their expression in mouse embryos. *Emx1* expression is detectable from day 9.5 of gestation whereas *Emx2* appears to be already expressed in 8.5 day embryos. Both genes are expressed in the presumptive cerebral cortex and olfactory bulbs. *Emx1* is expressed exclusively there, whereas *Emx2* is also expressed in some neuroectodermal areas in embryonic head including olfactory placodes in earlier stages and olfactory epithelia later in development.

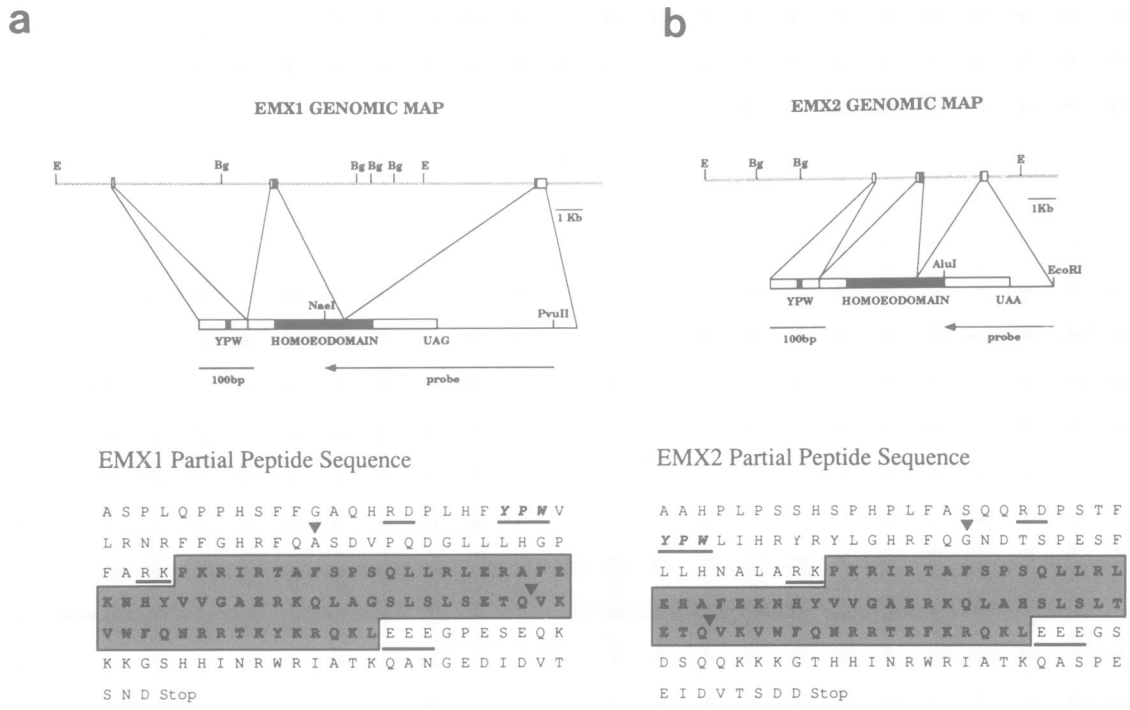
Key words: brain/development/gene expression/homeobox/olfaction

Introduction

Recent molecular approaches have contributed to identify genes that could be involved in vertebrate pattern formation. In particular, a considerable amount of knowledge has been recently gained about the genetic control of the identity of specific regions along the body axis of vertebrates (Kessel and Gruss, 1990). This was due primarily to the study of vertebrate homologues of *Drosophila* regulatory genes (Akam, 1987). Several such genes contain a homeobox, a conserved DNA sequence encoding a DNA binding domain termed homeodomain (Levine and Hoey, 1988; Gehring *et al.*, 1990). Through the recognition properties of their homeodomain, homeoproteins encoded by homeobox genes are believed to regulate the expression of batteries of target genes. At least seven classes of homeodomains have been extensively studied in *Drosophila*, known as *Antennapedia* (*Antp*), *bicoid* (*bcd*), *caudal* (*cad*), *engrailed* (*en*), *even-skipped* (*eve*), *muscle segment* (*msh*) and *paired* (*prd*) type homeodomains (Scott *et al.*, 1989). Murine homologues of all these classes have been described, with the exception of the *bcd* type (Scott *et al.*, 1989; Kessel and Gruss, 1990). Among these, the gene families characterized by a *prd*-like (*Pax*) or *Antp*-like (*Hox*) homeobox have been most systematically investigated (Kessel and Gruss, 1990). For example, *Hox* genes have been shown to be homologous (Boncinelli *et al.*, 1989; Duboule and Dollé, 1989; Graham *et al.*, 1989) (Akam, 1989 for a review) to the *Drosophila* homeotic genes belonging to the ANT-C and BX-C complexes present in flies where they are known to control segment identity along the major rostro-caudal body axis (Akam, 1987). The current data suggest that in many embryonic contexts the vertebrate *Hox* network is part of an evolutionarily conserved mechanism for specifying regional differences along the embryonic axis (Wilkinson *et al.*, 1989; Kessel *et al.*, 1990; Hunt *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991; Kessel and Gruss, 1991). Inspection of the phenotype of null mutations for *Hox-1.5* (Chisaka and Capecchi, 1991) and *Hox-1.6* (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992) in transgenic mice confirms this conclusion (Hunt and Krumlauf, 1991 for a review).

On the other hand, very little is known about the development of most anterior regions of the body, both in flies and vertebrates. In contrast to the case of thoracic and abdominal development, hierarchy of zygotically expressed *Drosophila* genes controlling head morphogenesis has not been clearly defined (Cohen and Juergens, 1991; Finkelstein and Perrimon, 1991). This is essentially due to the fact that understanding of the formation of the complex embryonic head region of flies has advanced quite slowly. Recently, however, substantial progress has been made in the identification and analysis of the genes determining head development (Dalton *et al.*, 1989; Cohen and Juergens, 1990; Finkelstein and Perrimon, 1990; Finkelstein *et al.*, 1990). Among them, three genes have been identified that appear to play a major role in controlling the development of the head, namely *empty spiracles* (*ems*) (Dalton *et al.*, 1989; Cohen and Juergens, 1990), *orthodenticle* (*otd*) (Finkelstein *et al.*, 1990) and *buttonhead* (*btd*) (Finkelstein and Perrimon, 1990). Interestingly, there is increasing evidence that the rules governing head formation may differ from the paradigm established for the central region of the body (Cohen and Juergens, 1991; Finkelstein and Perrimon, 1991). In fact, these three genes seem to be required both to establish contiguous blocs of segments and to specify segmental identity in the head. That is, they would share the properties of both gap and homeotic selector genes operating in the trunk of *Drosophila* embryos (Finkelstein and Perrimon, 1990).

ems (Dalton *et al.*, 1989) and *otd* (Finkelstein *et al.*, 1990) have been cloned and shown to contain a homeobox. It seemed of interest to look for vertebrate homologues of these genes and we undertook this scrutiny beginning with *ems*. Named because it is required for the development of the tracheal system in abdominal segment 8, *ems* mutations also result in the deletion of specific anterior head structures (Dalton *et al.*, 1989). At the blastoderm stage, the *ems* protein is expressed in a fairly anterior circumferential stripe. This stripe is under the regulation of the maternal *bcd* product, as embryos with varying dosage of *bcd* form the *ems* stripe at different antero-posterior positions (Dalton *et al.*, 1989). Later, the *ems* protein becomes localized to specific head regions of the extended germ band embryo. A homologous, genetically linked gene, termed E5, has also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EMX1 GENOMIC MAP
E
Bg
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1 Kb
NaeI
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EcoRI
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EMX1 Partial Peptide Sequence
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F A R K P K R I R T A F S P S Q L L R L E R A F E
K W H Y V V G A E R K Q L A G S L S L S E T Q V K
V W F Q N R R T K Y K R Q K L E E E G P E S E Q K
K K G S H H I N R W R I A T K Q A N G E D I D V T
S N D Stop
EMX2 Partial Peptide Sequence
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Y P W L I H R Y R Y L G H R F Q G N D T S P E S F
L L H N A L A R K P K R I R T A F S P S Q L L R L
E H A F E K N H Y V V G A E R K Q L A H S L S L T
E T Q V K V W F Q N R R T K F K R Q K L E E E G S
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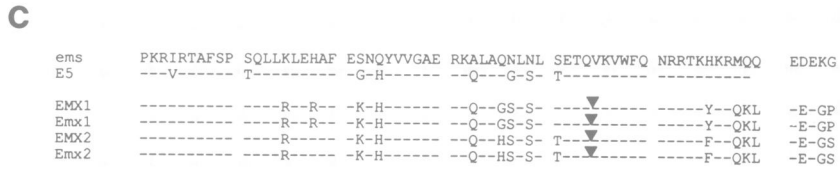
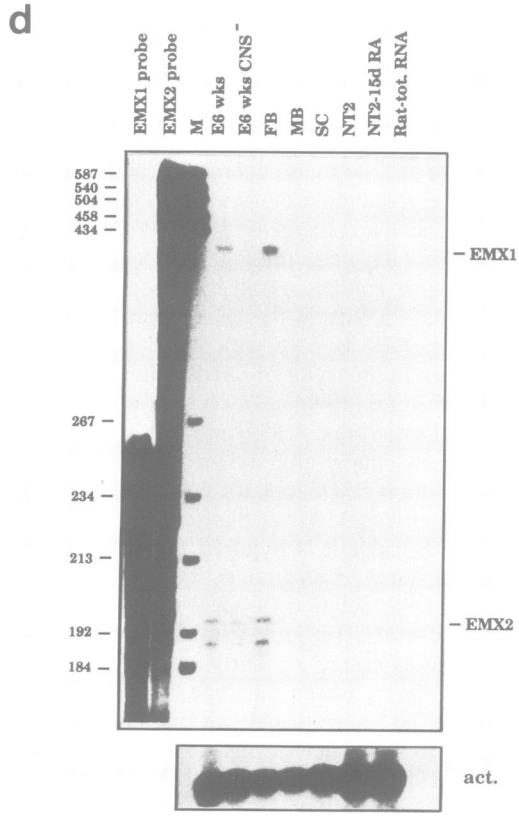

c
ems PKRIRTAFSP SQLLKLEHAF ESNQYVVGAE RKALAQNLNL SETQVKVWFQ NRRTKHKRMQQ EDEKG
E5 ---V------ T--------- -G-H------ --Q---G-S- T---------- ----------
EMX1 ---------- ----R--R-- -K-H------ --Q--GS-S- ----Y----- -----Y--QKL -E-GP
Emx1 ---------- ----R--R-- -K-H------ --Q--GS-S- ----Y----- -----Y--QKL -E-GP
EMX2 ---------- ----R----- -K-H------ --Q--HS-S- T---F----- -----F--QKL -E-GS
Emx2 ---------- ----R----- -K-H------ --Q--HS-S- T---F----- -----F--QKL -E-GS


d
EMX1 probe
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E6 wks
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540
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267
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213
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EMX1
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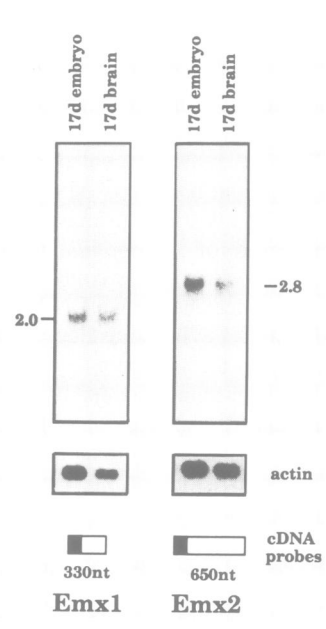
act.


e
17d embryo
17d brain
17d embryo
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2.0
2.8
actin
cDNA probes
330nt
650nt
Emx1
Emx2

been cloned. Its physical linkage to *ems*, however, has not been documented and its expression pattern is poorly characterized (Dalton *et al.*, 1989).

We cloned two homeobox genes, *Emx1* and *Emx2*, related to *ems* and studied their expression in mouse embryos. *Emx1* expression is detectable from day 9.5 of gestation whereas *Emx2* appears to be already expressed in 8.5 day embryos. Both genes are expressed in the presumptive cerebral cortex and olfactory bulbs. *Emx1* is expressed almost exclusively there, whereas *Emx2* is also expressed in some neuroectodermal areas in embryonic head including olfactory placodes in earlier stages and olfactory epithelia later in development.

Results

Isolation of ems-like clones

We screened a cDNA library prepared from 8 week human embryos (Acampora *et al.*, 1989) with an *ems* genomic sequence including the homeobox and found two homologous cDNA clones, we termed *EMX1* and *EMX2*, containing a homeodomain very similar to that of *ems* (Figure 1). Using these cDNA clones as probes we screened in turn a genomic library constructed in cosmids (Acampora *et al.*, 1989) and compared corresponding regions in cDNA and genomic clones. The exon−intron organization and the peptide sequence of relevant regions of *EMX1* and *EMX2* are shown in Figure 1(a and b), respectively. Figure 1(c) shows a comparison of the homeodomains of *ems*, E5, *EMX1* and *EMX2*. *EMX1* and *EMX2* homeodomains differ for four amino acid residues from each other and both differ for 11 residues from the *ems* homeodomain. In particular, there is no amino acid difference in the third, recognition, helix of the four homeodomains. The similarity of the putative products of these genes with *ems* is not confined to the homeodomain. It is extended downstream from it for three acidic residues and upstream from it for two residues, i.e. Arg-Lys. Homology to *ems* includes also a short protein domain located further upstream from the homeodomain, namely Arg-Asp-X-X-X-H-Tyr-Pro-Trp-H-H, where X is any amino acid and H is a hydrophobic residue (Dalton *et al.*, 1989) (Figure 1). This motif includes a divergent version of the conserved homeopentapeptide Ile/Phe-Tyr-Pro-Trp-Met present in several homeotic genes of *Drosophila* and in most vertebrate genes belonging to the HOX clusters (Boncinelli *et al.*, 1991) (Figure 1a and b). An intron is present in both *EMX1* and *EMX2* between the exons containing the homeopentapeptide and the homeodomain, as is often the case for homeobox genes. An additional intron is present in both genes within the homeobox at identical positions, namely residue 44 of the homeodomain. No evidence for a similar intron has been reported for *ems* (Dalton *et al.*, 1989).

Thus *EMX1* and *EMX2* appear to represent two human homologues of the *Drosophila ems* gene (as well as of E5). We investigated *EMX1* and *EMX2* expression in human 6 week embryos by means of an RNase protection assay of RNA extracted from three regions of the embryonic central nervous system (CNS) (Figure 1d). A protection signal can be detected in the forebrain confirming the expectation that these two vertebrate genes should exhibit an anterior domain of expression. Conversely, neither gene was significantly expressed in embryonal carcinoma NT2/D1 cells (Simeone *et al.*, 1990) even after retinoic acid (RA) treatment (Figure 1d). This observation stresses the difference between these genes and the homeobox genes of the HOX loci, most of which are activated in these cells upon retinoic acid treatment (Simeone *et al.*, 1990, 1991) (Boncinelli *et al.*, 1991 for a review).

We then decided to study their expression domains in mouse embryos. Using the human cDNA clones as probes we screened a cDNA library prepared from 11 day mouse embryos (Clontech) and isolated corresponding mouse clones, termed *Emx1* and *Emx2*. *Emx1* and *Emx2* homeodomains are identical to their human cognates (Figure 1c). Figure 1(e) shows a Northern blot analysis of polyadenylated RNA from 17 day mouse embryos. Single transcripts 2 kb and 2.8 kb long are detected by *Emx1* and *Emx2* probes, respectively, in the brain at this developmental stage.

Expression domains in 12.5 d.p.c. embryos

Emx1. We first analysed 12.5 d.p.c. (days post coitum) mouse embryos hybridizing sagittal (Figure 2), frontal and transverse (Figure 3) sections with the murine probes shown in Figure 1(e). *Emx1* appears to be expressed in extended regions of the dorsal telencephalon. In sagittal sections (Figure 2a−e), *Emx1* expression is detectable in the presumptive cortex from its posterior boundary to its anterior boundary and includes the olfactory bulb (Figure 2d). An enlargement of the cortical region is shown in Figure 2(k). Hybridization signal is uniformly distributed across the cortex, without major differences. Sagittal sections in the middle of lateral ventricles reveal expression in the olfactory bulbs. No *Emx1* expression is detectable in non-cortical basal telencephalic regions. A very similar expression pattern is observed in sagittal sections of 13.5 d.p.c. embryos (Figure 2n and o) apart from the fact that the cross section

Fig. 1. Structure and expression of *Emx1* and *Emx2*. (**a**) Cloned cDNA region and genomic organization of the human *EMX1* gene. The probe used in RNase protection experiments is shown below the cDNA scheme. The deduced peptide sequence of the cloned cDNA region is also shown, using the one-letter amino acid code. The homeodomain is boxed. Conserved peptide motifs (see text) are underlined and arrowheads point to splice sites. (**b**) Cloned cDNA region and genomic organization of *EMX2*. The *Eco*RI site at the 3′ end of the cDNA clone belongs to the vector. (**c**) Comparison of *EMX1*, *EMX2*, *Emx1* and *Emx2* homeodomains with *ems* and E5 homeodomains. Five amino acid residues following the homeodomain are also shown. Dashes indicate amino acid identity with *ems* and arrowheads point to splice sites. (**d**) RNase protection experiments with human *EMX1* and *EMX2* genes. Total RNA (20 μg) from total 6 week embryos, 6 week embryos deprived of the CNS (CNS$^-$), dissected forebrain (FB), midbrain (MB) and hindbrain and spinal cord (SC) was hybridized to the RNA probes indicated in (a) and (b) and RNase digested. Human β-actin control is also shown. Both genes appear to be significantly expressed only in total embryos or in the embryonic forebrain. RNA from human embryonal carcinoma NTera2/clone D1 cells untreated (NT2) and after 15 days of 10 μM retinoic acid treatment (NT2-15d RA) was also analysed. In neither case was a protection signal observed. (**e**) Expression of murine *Emx1* and *Emx2* in 17 day mouse embryos. Northern blot analysis of polyadenylated RNA (7 μg) extracted from total embryos and from brains of the same gestational age hybridized with the probes indicated. Approximate size of *Emx1* and *Emx2* transcripts is indicated in kb.

of the cortical region has grown considerably with respect to the 12.5 d.p.c. developmental stage. A single non-cortical localization of *Emx1* expression can be observed in Figure 2(n and o), confined to a spot (arrowhead) probably corresponding to a periventricular complex at the posterior boundary of diencephalon (see also below).

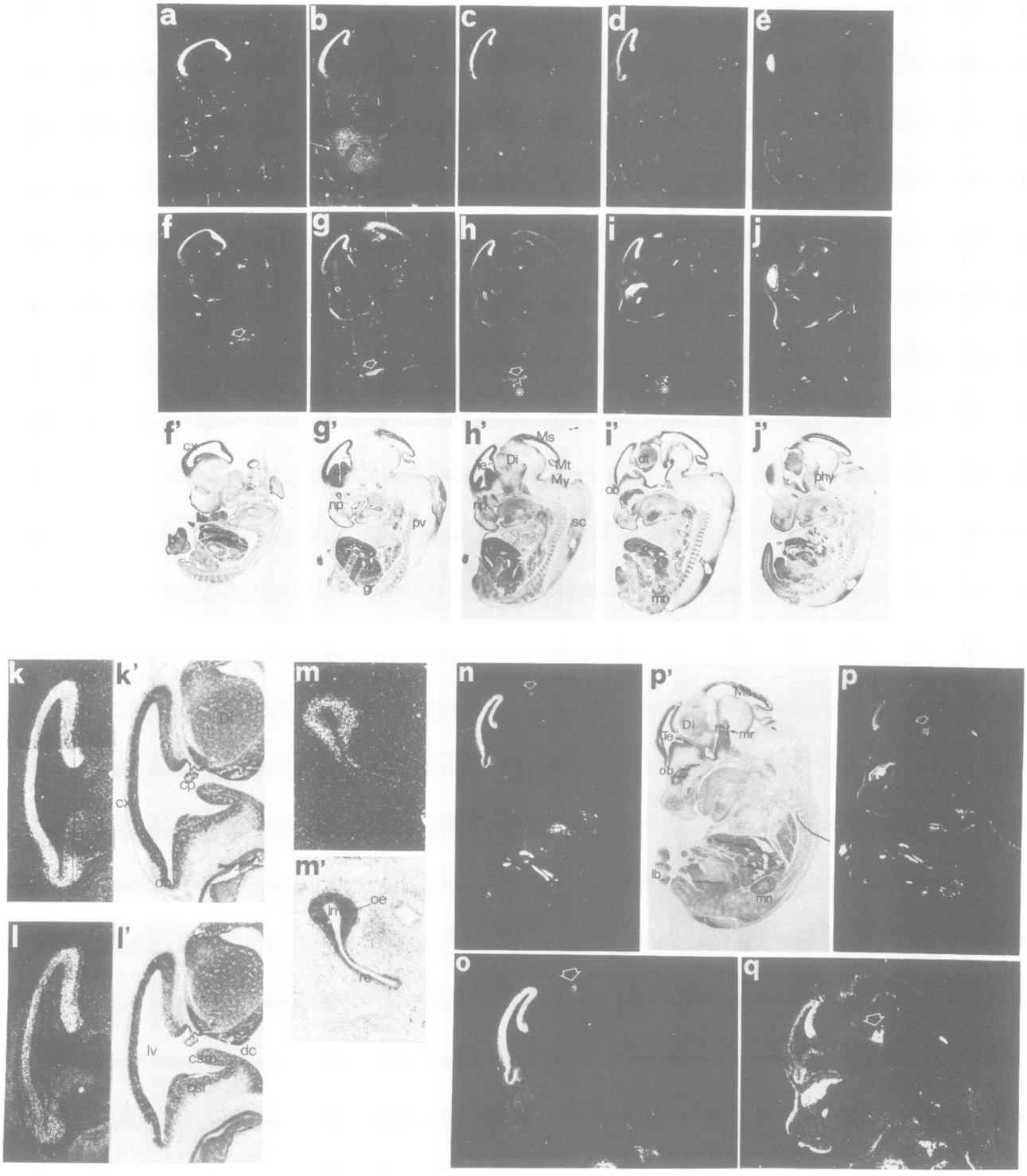

Fig. 2. *Emx1* and *Emx2* expression in sagittal sections of 12.5 d.p.c. (**a**)−(**m**) and 13.5 d.p.c. (**n**)−(**q**) mouse embryos. (a−e), (k) and (n) and (o) show hybridization with *Emx1*. (f)−(j), (l) and (m) and (p) and (q) show hybridization with *Emx2*. Sections (a)−(e) and (f)−(j) are progressively more medial. An arrowhead in (f)−(h) points to the genital ridge, whereas in (n) and (o) it points to the single non-cortical *Emx1* expression site and in (p) and (q) indicates *Emx2* expression in mammillary recess. A filled arrowhead in (p) points to limb ectoderm. An asterisk in (h) and (i) indicates presumptive metanephric epithelium. A bright field exactly corresponding to a given dark field is indicated by a prime affix; e.g. the section corresponding to the (z) dark field is indicated as (z'). (m) shows an enlargement of *Emx2* expression in nasal pits shown in (g). cp, choroid plexus; cs, corpus striatum; csl, corpus striatum laterale; csm, corpus striatum mediale; cx, cortex; dc, diocoel; Di, diencephalon; dt, dorsal thalamus; gr, genital ridge; lb, limb; lm, lumen; lv, lateral ventricle; mn, metanephros; mr, mammillary recess; Ms, mesencephalon; Mt, metencephalon; My, myelencephalon; np, nasal pits; ob, primordium of olfactory bulb; oe, olfactory epithelium; phy, posterior hypothalamus; pv, prevertebrae; re, respiratory epithelium; sc, spinal cord; Te, telencephalon.

The cortical expression of *Emx1* in its lateral to medial extension is better seen in frontal sections of 12.5 d.p.c. mouse embryos (Figure 3a–d). In particular, section 3(a) localizes the single non-cortical *Emx1* expression domain in presumptive periventricular nuclei.

In transverse sections the cortical nature of *Emx1* expression is even more apparent (Figure 3m–o). These hybridization data confirm the restricted localization of the *Emx1* expression domain in dorsal regions of lateral ventricles including the hippocampal and parahippocampal areas as well (Figure 3m and n). Figure 3(o) further localizes the non-cortical spot of *Emx1* expression.

Emx2. Let us consider now *Emx2* expression in 12.5 d.p.c. mouse embryos. Sagittal sections (Figure 2f–j) reveal that *Emx2* is expressed in a subset of the *Emx1* expression domains in the presumptive cortex. At this stage the hybridization signal is much stronger in the posterior dorsal telencephalon with a sharp posterior boundary and decreasing intensity in progressively more anterior regions (see a more detailed picture in Figure 2l). An appreciable hybridization signal is, however, present in olfactory bulbs (Figure 2l and q). The intensity of *Emx2* expression is not as uniformly distributed across the cortical layers (Figure 2l) as the *Emx1* expression. *Emx2* expression is almost undetectable within the external mantle layer. Like *Emx1*, no *Emx2* expression is detectable in internal basal telencephalic regions. Conversely, *Emx2* is not expressed only in the cortex. Its expression domain in the head appears to be extended to specific diencephalic regions (Figure 2h–j), to limited

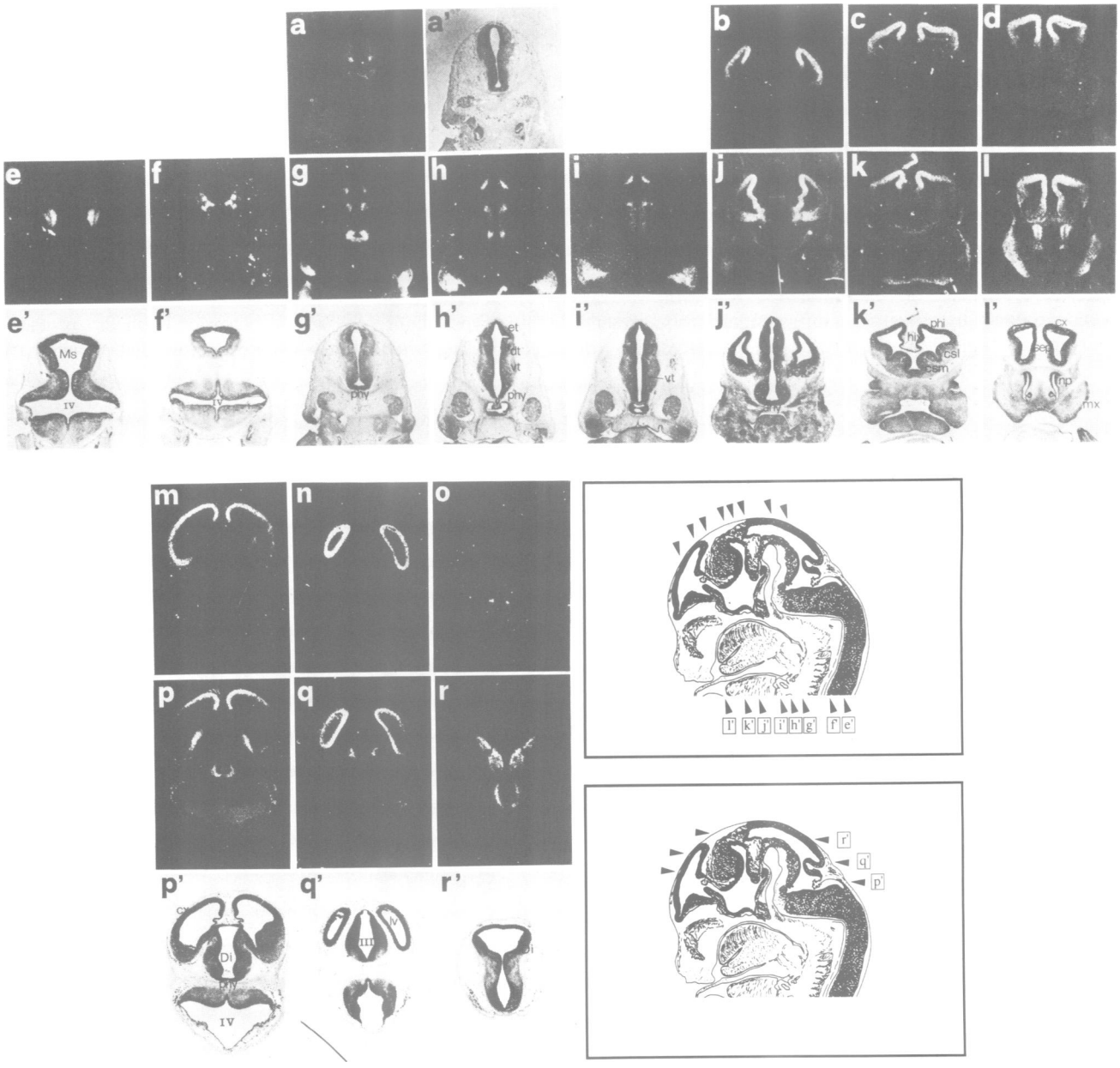

Fig. 3. *Emx1* and *Emx2* expression in frontal (**a**)–(**l**) and transverse (**m**)–(**r**) sections of 12.5 d.p.c. mouse embryos. (a)–(d) and (m)–(o) show hybridization with *Emx1*. (e)–(l) and (p)–(r) show hybridization with *Emx2*. Symbols as in Figure 2 and: ahy, anterior hypothalamus; bcx, posterior basal telencephalic cortex; et, epithalamus; hix, primordium of hippocampal cortex; mx, maxillary process; phi, primordium of the parahippocampal cortex; sep, primordium septi; vt, ventral thalamus; III and IV, third and fourth ventricle. Schemes of the various sections are also shown.

mesencephalic areas (Figure 2g–j) and to epithelia in the nasal cavities (Figure 2g–i). Figure 2(m) shows that the olfactory and not the respiratory epithelia in nasal pits display *Emx2* expression (see also below). In this regard it may be of interest to note that *ems* itself is involved in the regulation of sense organs during development of *Drosophila* antennal segment, because this pair of organs is missing in developing mutant *ems* flies (Dalton *et al.*, 1989). Antennal sense organs are considered the main olfactory sensory structures of the *Drosophila* larva.

Emx2 is also expressed in ectodermal regions in the snout (Figure 2f–j) as well as in metanephric tubular epithelia (Figure 2h and i, asterisk) and in the genital ridge (Figure 2f–h, arrowhead). A similar expression pattern is observed in sagittal sections of 13.5 d.p.c. embryos (Figure 2p and q). Figure 2(q) better illustrates the uneven *Emx2* distribution across the cortical layers and its localization in diencephalon (arrowhead). Figure 2(p and q) clearly shows that *Emx2* is also expressed in nasal cavities and ectodermal regions in the snout and limbs (filled arrowhead in Figure 2p) as well as in metanephric tubular epithelia (Figure 2p).

The diverse *Emx2* expression in the head is confirmed in frontal and horizontal sections (Figure 3). Figure 3(e and f) show *Emx2* expression in localized mesencephalic (Figure 3e) and possibly interpeduncular areas (Figure 3f), whereas Figure 3(g–i) show its localization in epithalamic, thalamic and hypothalamic regions. Thalamic expression in sections (g)–(i) is strong in a limited region of the ventral portion of dorsal thalamus, but a diffuse signal is also present in ventral thalamus (Figure 2h–i). Expression in hypothalamic regions just posterior to the localization of the Rathke's pouch is particularly evident in Figure 3(g). Intensity distribution in diencephalon varies with the antero-posterior level of the section (compare sections g, h and i). Expression in dorsal (Figure 3j–l) and posterior basal (Figure 3j) cortical regions is also apparent. Finally, *Emx2* expression is detectable in branchial arches. In Figure 3(g–i), expression in the first, mandibular, arch is observable.

Transverse sections (Figure 3p–r) demonstrate that the major site of *Emx2* expression is in the cortex. As previously noticed, additional *Emx2* expression sites can be detected in diencephalon (Figure 3p–r) and mesencephalon (Figure 3r).

Early expression

The picture emerging from *in situ* hybridization analysis on 12.5 d.p.c. embryos points to a restricted cortical localization of *Emx1* expression and of at least part of *Emx2* expression. We further investigated the temporal profile of their expression. *Emx1* expression is first detectable in 9.5 d.p.c. embryos while *Emx2* expression is detectable earlier, in 8.5 d.p.c. embryos (Figure 4).

Emx1 is first expressed in the anterior dorsal region of the neural tube (Figure 4a), an area fated to give rise to telencephalic cortical regions, at a stage when regionalization is probably already specified and cortical neurogenesis is just starting (Luskin *et al.*, 1988). Antero-posterior delimitation of the *Emx1* expression domain is more evident in 9.75 d.p.c. embryos (Figure 4b) with a posterior boundary probably coinciding with that between presumptive diencephalon and

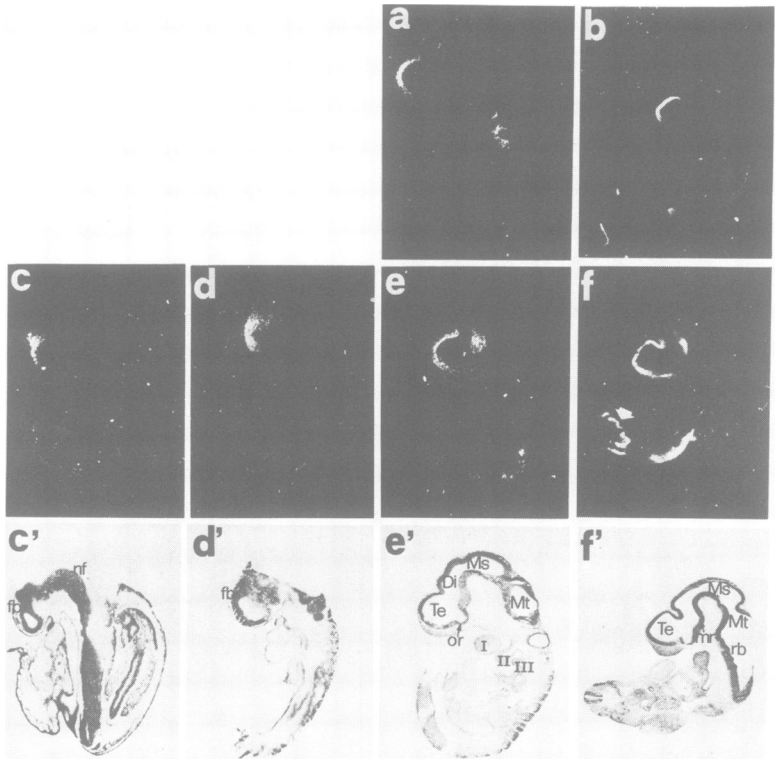

Fig. 4. Early *Emx1* and *Emx2* expression in sagittal sections of 8.5 d.p.c. (**c**), 9 d.p.c. (**d**), 9.5 d.p.c. (**a** and **e**) and 9.75 d.p.c. (**b** and **f**) mouse embryos. (a)–(b) show hybridization with *Emx1*. (c)–(f) show hybridization with *Emx2*. A filled arrowhead in (f) points to the *Emx2* expression site in the coelomic epithelium covering the mesonephric column as well as the final part of the mesenteric attachment. Symbols as in Figure 2 and: fb, forebrain; nf, neural fold; or, optic recess; rb, rhombomeres; I, II and III, first, second and third branchial arches.

telencephalon (see also Figure 6). A hybridization signal is also present on branchial ectoderm in 9.5 d.p.c. embryos but not in 9.75 d.p.c. embryos (Figure 4a and b).

Emx2 is expressed at an earlier developmental stage than *Emx1*. A hybridization signal is already detectable in anterior dorsal neuroectodermal regions in 8.5 d.p.c. embryos (Figure 4c). *Emx2* hybridization signal is stronger in 9 d.p.c. embryos (Figure 4d). Antero-posterior delimitation of the *Emx2* expression in dorsal neuroectoderm is clear in 9.5 d.p.c. embryos (Figure 4e) and even more evident in 9.75 d.p.c. embryos (Figure 4f) with an anterior boundary probably identical to that displayed by *Emx1* and a posterior boundary well within the roof of presumptive diencephalon. Ventral expression in the floor of presumptive diencephalon is also evident at this developmental stage, prefiguring later expression in various diencephalic areas (Figure 3), whereas no expression is detectable in the mesencephalon at this stage. An anterior ectodermal localization of *Emx2* expression is also observable in 9.75 d.p.c. embryos in a continuous region probably including olfactory placodes (Figure 4f). *Emx2*

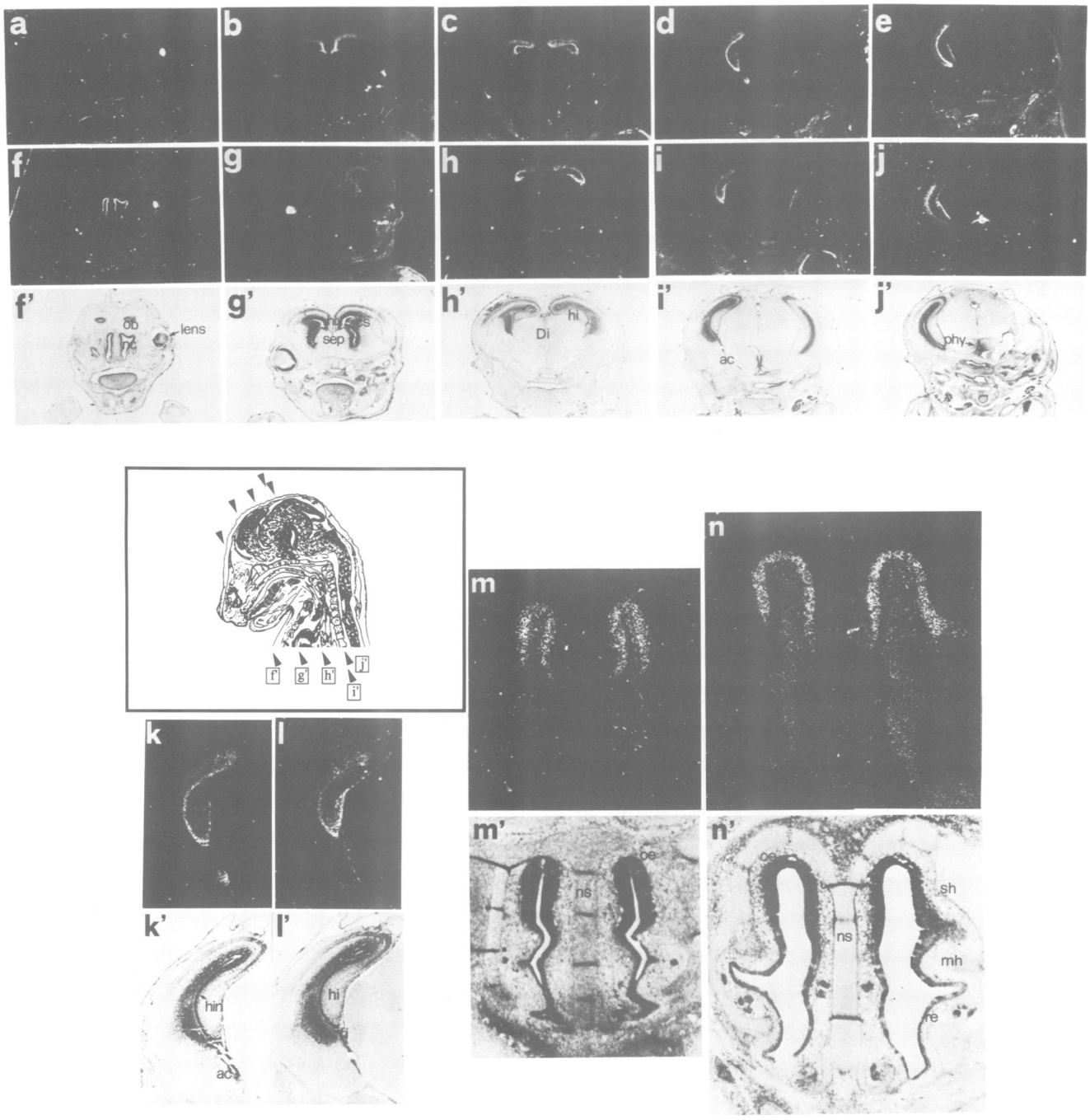

Fig. 5. *Emx1* and *Emx2* expression in frontal sections of the head of 17 d.p.c. mouse embryos. (**a**)–(**e**) and (**k**) show hybridization with *Emx1*. (**f**)–(**j**), (l) and (**m**)–(**n**) show hybridization with *Emx2*. (k) and (l) show details of the hippocampal region of sections (d) and (i), respectively. (m) and (n) show *Emx2* expression in nasal cavities (Croucher and Tickle, 1989) of 13.5 d.p.c. and 17 d.p.c. embryos, respectively. Symbols as in Figure 2 and: ac, amigdaloid complex primordium; fi, fimbria; hi, hippocampus; hin, proliferating hippocampal neuroepithelium; hix, primordium of the precommissural hippocampal cortex; mh, middle concha; ns, nasal septum; phi, parahippocampal cortex; sep, septum; sh, superior concha. A scheme of the various sections is also shown.

expression is also detectable at this stage in the coelomic epithelium covering the mesonephric column as well as the final part of the mesenteric attachment (Figure 4f, filled arrowhead), fated to contribute to the genital ridge in later stages of development (see above, Figure 2f–h).

Late expression

Expression patterns of *Emx1* and *Emx2* persist almost identical in 13.5 and 15 d.p.c. embryos (not shown). Figure 5 shows their expression in cephalic regions of 17 d.p.c. embryos.

Emx1 is still expressed in olfactory bulbs, whereas a new expression site is now the developing lens (Figure 5a). *Emx1* cortical expression is much reduced in intensity and extension though still persisting in growing hippocampal germinal layer (Figure 5c–e and k) and in the primordium of the amigdaloid cortical complex (Figure 5d and k).

Emx2 expression (Figure 5f–j and l) reproduces basically the *Emx1* expression pattern but includes also olfactory epithelia in nasal cavities (Figure 5f and n) and posterior hypothalamic germinal areas (Figure 5j). A comparison of *Emx2* expression in nasal cavities at 13.5 d.p.c. and 17 d.p.c. is shown in Figure 5(m and n).

Discussion

We cloned two homeobox genes related to the *Drosophila ems* and E5 genes (Dalton *et al.*, 1989). Both in humans and mouse they contain an intron within the homeobox, at the level of the 44th residue of the homeodomain (Figure 1a and b). Both are expressed in embryonic cerebral cortex (Figures 2 and 3) in a developmental period, between day 10 and day 16 post coitum, corresponding to major events in cortical neurogenesis. At day 17 of development, cortical expression of the two genes is almost exclusively confined to hippocampal germinal layers (Figure 5). Several homeobox genes are believed to control cell identity with a regional or even segmental pattern (Kessel and Gruss, 1990; Price *et al.*, 1991). Henceforth it seems reasonable to speculate about a possible role of *Emx1* and *Emx2* in establishing the limits and identity of the embryonic cerebral cortex.

Temporal patterns of neurogenesis are believed to be important prerequisites for the establishment of precise anatomical interactions in the developing brain. The two genes are expressed in most cortical regions with a precise temporal profile. In its full extension, 12.5 to 13.5 d.p.c., the *Emx1* expression domain comprises cortical regions including primordia of neopallium, hippocampal and parahippocampal archipallium (Kuhlenbeck, 1973) (Figure 6a). *Emx1* expression seems characteristic of cortical regions, mainly but not exclusively hexalaminar in nature. Its expression progressively declines in anterior and external cortical regions and at day 17 post coitum is confined to germinal hippocampal layers. It is of interest to note that neurogenesis of pyramidal neurons is still on at this developmental stage whereas neurogenesis of other cell types in hippocampal regions takes place even later (Stanfield and Cowan, 1979). It would be tempting to speculate on a role of this gene during cortical neurogenesis. Similarly, the *Emx2* expression domain comprises presumptive cortical regions including neopallium, hippocampal and parahippocampal archipallium and selected palaeopallial localizations (Figure 6a), but no basal internal grisea (Kuhlenbeck, 1973).

The temporal profiles of expression of the two genes are very similar but not coincident. There is a temporal shift between *Emx1* and *Emx2* expression (Figure 6b). The latter is expressed earlier (Figure 4) and declines earlier in anterior cortical regions (Figure 2). Its first expression roughly coincides with the appearance of neuromere subdivision 4 (Sakai, 1987) dividing forebrain from midbrain and precedes the appearance of subdivision 2 dividing telencephalon from diencephalon (Sakai, 1987) (Figure 6b). After the appearance of this subdivision, *Emx1* expression begins to be detectable in dorsal telencephalon. *Emx2* is expressed in dorsal telencephalon but also in restricted regions of diencephalon, anterior dorsal and posterior ventral, both before and after the appearance of subdivision 3 which divides diencephalon (Figure 6b). Later on (Figures 2 and 3) *Emx2* is also expressed in some mesencephalic regions. Neither gene is expressed in hindbrain or spinal cord.

There is only a single non-cortical hybridization site of *Emx1* lying at both sides of the third ventricle in a

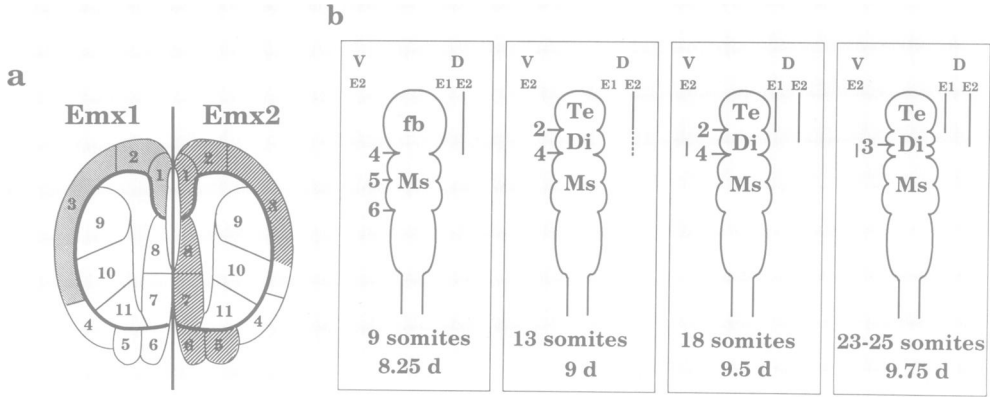

Fig. 6. *Emx1* and *Emx2* expression in the embryonic CNS. **(a)** Idealized scheme (Kuhlenbeck, 1973) of a telencephalic cross-section showing primordia of cortical grisea (1–6) and of internal grisea (7–11). Hatching indicates *Emx1* expression on the left side and *Emx2* expression on the right side. *Emx2* expression in regions 5 and 6 is only detectable in more posterior sections, and *Emx2* expression in regions 7 and 8 is clearly detectable in more anterior sections. 1, hippocampal cortex; 2, parahippocampal cortex; 3, neocortex; 4, piriform cortex; 5, regio insularis of neocortex; 6, basal cortex; 7–8, pre- and para-terminal complex (septum); 9–10–11, primordia of basal ganglia. **(b)** Early expression of *Emx1* and *Emx2* in dorsal (D) and ventral (V) brain regions. Numbers indicate neuromere subdivisions according to Sakai (1987). fb, forebrain; Di, diencephalon; Ms, mesencephalon; Te, telencephalon.

periventricular location (Figures 2n–o and 3a and o). Conversely, *Emx2* is expressed in several neuroectodermal regions of the embryo. Particularly interesting is its expression in olfactory epithelia of nasal chambers and in several locations related to olfaction. In fact, *Emx2* is expressed in primordia of olfactory bulbs, hippocampal and parahippocampal cortex, amigdalae, specific areas of basal cortex, hypothalamus, ventral and dorsal thalamus, habenulae, mammillary bodies, septal and tegmental regions. All these regions contain areas related to olfaction. Some of these are primary olfactory complexes while others represent centres of secondary elaboration of olfactory stimuli, as, for example, diencephalic structures such as habenulae and hypothalamus. The habenulae are in communication with the amigdalae and the olfactory tuberculi, and the hypothalamus, including the mammillary region, is in communication with the hippocampus and the olfactory tuberculi. In addition, specific mesencephalic tegmental areas receive descending projections from both the hypothalamus and the habenulae (Kuhlenbeck, 1973). It remains to be seen whether *Emx2* expression sites coincide with primordia of these areas as is the case for olfactory epithelia in nasal pits and chambers. The idea that homeobox genes of the *ems* family might be involved in the specification of the proto-olfactory system seems intriguing.

It has been proposed that the body of jawed vertebrates may be considered as subdivided into three main regions, the preotic head, the branchial area and the trunk (Gans and Northcutt, 1983). It is of interest to speculate as to what extent this proposal corresponds to molecular data about homeobox gene expression. In the trunk, *Hox* genes located in the 5′ and central portion of the *Hox* gene clusters are expressed in a complex pattern where offsets in expression of corresponding genes of the four clusters mark particular somites and parts of the spinal cord (Wilkinson *et al.*, 1989; Kessel and Gruss, 1990, 1991; Kessel *et al.*, 1990; Hunt *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991). In developing branchial areas, *Hox* genes located in the 3′ regions of *Hox* loci are expressed with an overlapping code of rhombomere and branchial arch specification (Wilkinson *et al.*, 1989; Hunt *et al.*, 1991). Finally, in preotic head, no *Hox* gene is expressed but other homeobox gene families (He *et al.*, 1989; Krauss *et al.*, 1991; Price *et al.*, 1991; Porteus *et al.*, 1991; Walther and Gruss, 1991), including the *Emx* gene family, appear to play a major role. How many gene families of this type are involved and the actual function they are playing in the developing head, remains to be investigated.

Materials and methods

cDNA and genomic screening

A cDNA library prepared from 8 week human embryos (Acampora *et al.*, 1989) was screened at low stringency conditions with a short *ems* genomic sequence including the homeobox. This *ems* genomic region was obtained by PCR amplification through a standard protocol of *Drosophila* total DNA using as primers two synthetic oligonucleotides derived from the published *ems* sequence (Dalton *et al.*, 1989). Two classes of homologous cDNA clone, termed *EMX1* and *EMX2*, were found. Using these cDNA clones as probes we screened in turn a human genomic library constructed in cosmids (Acampora *et al.*, 1989) and compared corresponding regions in cDNA and genomic clones to study the transcriptional organization of the two genes. Using the human cDNA clones as probes we screened a cDNA library prepared from 11 day mouse embryos (Clontech) and isolated corresponding mouse clones, termed *Emx1* and *Emx2*.

Mice and cells

C57/Bl6 mice were mated between 9 p.m. and 10 a.m. Day 0.5 p.c. was assumed to begin at the middle of the day of vaginal plugging. Embryos of 8.5, 9, 9.5 and 9.75 d.p.c. have 8, 15, 22–23 and 27 somites, respectively (Sakai, 1987). Pregnant female mice were killed by cervical dislocation and embryos were collected in ice-cold PBS under a dissection microscope (Zeiss SV11) and fixed in 4% paraformaldehyde overnight. Human embryonal carcinoma cells of the NTERA-2 line, clone D1, (NT2/D1) were cultured and treated as previously reported (Simeone *et al.*, 1991).

Preparation of ^{32}P- and ^{35}S-labelled RNA probes for Emx1 and Emx2 for RNase protection and in situ hybridization

Emx1 and *Emx2* sense and antisense RNA probes were synthetically produced using respectively a 330 nucleotide *Pvu*II fragment and a 650 nucleotide *Alu*I–*Eco*RI fragment as templates (Figure 1e). Plasmids containing the insert of interest, and flanking T7 or Sp6 promoter sequences (pGEM3, Promega Biotec) were linearized with appropriate enzymes.

For RNase protection analysis, antisense strand transcription reactions, with T7 or Sp6 polymerase (Riboprobe Kit, Promega) were carried out in the presence of [^{32}P]GTP (Amersham).

For *in situ* hybridization, transcription reactions with T7 or Sp6 polymerase (Riboprobe Kit, Promega Biotec) were carried out in presence of [^{35}S]CTP (Amersham). The template was then degraded with RNase-free DNase (Pharmacia), and the labelled RNA was purified through a Sephadex G-50 column. The transcripts were progressively degraded to an average length of 150 nucleotides by random alkaline hydrolysis, to improve access to RNA *in situ*. The probes were dissolved at a working concentration of 1×10^5 c.p.m./μl in hybridization mix (Wilkinson and Green, 1989).

RNase protection and Northern blot

RNase protection and Northern blot experiments were performed following standard protocols (Simeone *et al.*, 1991).

In situ hybridization

In situ hybridization was carried out as described by Wilkinson and Green (1990) with minor modifications. 30 μl of the appropriate probe in hybridization mix was added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (65°C, 2×SSC, 50% formamide) and treated with RNase to remove unhybridized and non-specifically bound probe. Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were between 5 and 12 days. After developing, sections were stained in 0.02% Toluidine blue and mounted in DPX. Sections were examined and photographed using a Zeiss SV11 microscope with both dark- and bright-field illumination.

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