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A vertebrate gene related to *orthodenticle* contains a homeodomain of the *bicoid* class and demarcates anterior neuroectoderm in the gastrulating mouse embryo

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We studied the expression of two vertebrate homeobox genes, *Otx1* and *Otx2*, related to *orthodenticle*, a gene expressed in the developing head of *Drosophila*. Both genes are expressed in restricted regions of the developing rostral brain including the presumptive cerebral cortex and olfactory bulbs. The expression patterns of the two genes in diencephalon suggest that they both have a role in establishing the boundary between presumptive dorsal and ventral thalamus. They are also expressed in regions of the developing olfactory, auricolar and ocular system, including the covering of the optic nerve. *Otx1* expression is detectable from day 8 of gestation in telencephalic, diencephalic and mesencephalic regions. From day 10.5 of gestation its expression extends to some metencephalic areas. *Otx2* appears to be already expressed in the epiblast of prestreak embryos. It persists in the entire embryonic ectoderm for some time after the onset of gastrulation. In midstreak embryos its expression appears progressively restricted to the anterior embryonic ectoderm corresponding to presumptive fore- and mid-brain. In early midgestation embryos it is expressed in telencephalic, diencephalic and mesencephalic regions but from day 11.75 of gestation its expression disappears from dorsal telencephalon and is confined to diencephalic and mesencephalic regions. *Otx2* is one of the earliest genes expressed in the epiblast and immediately afterwards is expressed in anterior neuroectoderm, demarcating rostral brain regions even before headfold formation. Its gene product contains a homeodomain of the *bicoid* class and is able to recognize and transactivate a *bicoid* target sequence.

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

Introduction

A considerable amount of knowledge has recently been gained about the genetic control of the identity of specific regions along the body axis of vertebrates (Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). This is primarily due to the study of vertebrate homologues of regulatory genes operating in the *Drosophila* trunk (Akam, 1987). Conversely, until very recently very little was known about the development of most anterior regions of the body, even in flies (Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991). Three *Drosophila* genes have now been identified that appear to play a major role in controlling the development of the head (Dalton *et al.*, 1989; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein *et al.*, 1990). Two of them, *empty spiracles* (*ems*: Dalton *et al.*, 1989; Cohen and Jürgens, 1990; Walldorf and Gehring, 1992) and *orthodenticle* (*otd*: Finkelstein *et al.*, 1990; Wieschaus *et al.*, 1992), have been cloned and shown to contain a homeobox. We used *Drosophila* sequences to look for vertebrate homologues. We previously cloned and characterized *Emx1* and *Emx2* (Simeone *et al.*, 1992b), two mouse genes related to *ems*, and reported a preliminary characterization of two homologues of *otd*, *Otx1* and *Otx2* (Simeone *et al.*, 1992a).

In homozygous embryos, lethal *otd* alleles cause pattern deletions in the antennal and preantennal regions of the head (Wieschaus *et al.*, 1984, 1992; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). In mutant *otd* embryos, precursor cells in these regions do not express genes characteristic of those segments, suggesting that *otd* is required for their proper developmental programming (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). At the blastoderm stage, *otd* is expressed in a circumferential stripe of cells at the anterior end of the egg (Finkelstein *et al.*, 1990). This early domain of expression includes the precursors for the regions affected in mutant embryos. *otd* encodes a homeodomain-containing protein and thus probably functions as a transcription factor during early development (Finkelstein *et al.*, 1990). It has been proposed that *otd* functions like a gap gene in defining antennal and preantennal segments in the head.

In addition to its early role in the anterior region of the embryo, *otd* is required later in development. Homozygous *otd* embryos show abnormalities in the specification of medial cells in the central nervous system (Finkelstein *et al.*, 1990; Klämbt *et al.*, 1991). It has been argued (Wieschaus *et al.*, 1992) that *otd* also plays a central role in the formation of medial structures in the embryonic epidermis and this in turn may be related to its role in patterning of the embryonic central nervous system. Finally, a group of mutations exists in flies that fail to complement *otd* mutations. Flies homozygous for these mutations, *ocelliless*, survive to adult stages but show deletions of sense organs, including ocelli, in the adult head. The ocellar defects are assumed (Wieschaus *et al.*, 1992) to reflect a requirement for *otd* during development of the eye-antennal disc.

We now report an extensive analysis of two mouse and human homologues of *otd*, *Otx1* and *Otx2*. Both genes are expressed in restricted regions of the developing rostral brain of mouse midgestation embryos, including the presumptive cerebral cortex and olfactory bulbs. *Otx2* appears to be already expressed in the epiblast of prestreak embryos. It persists in the entire embryonic ectoderm for some time after

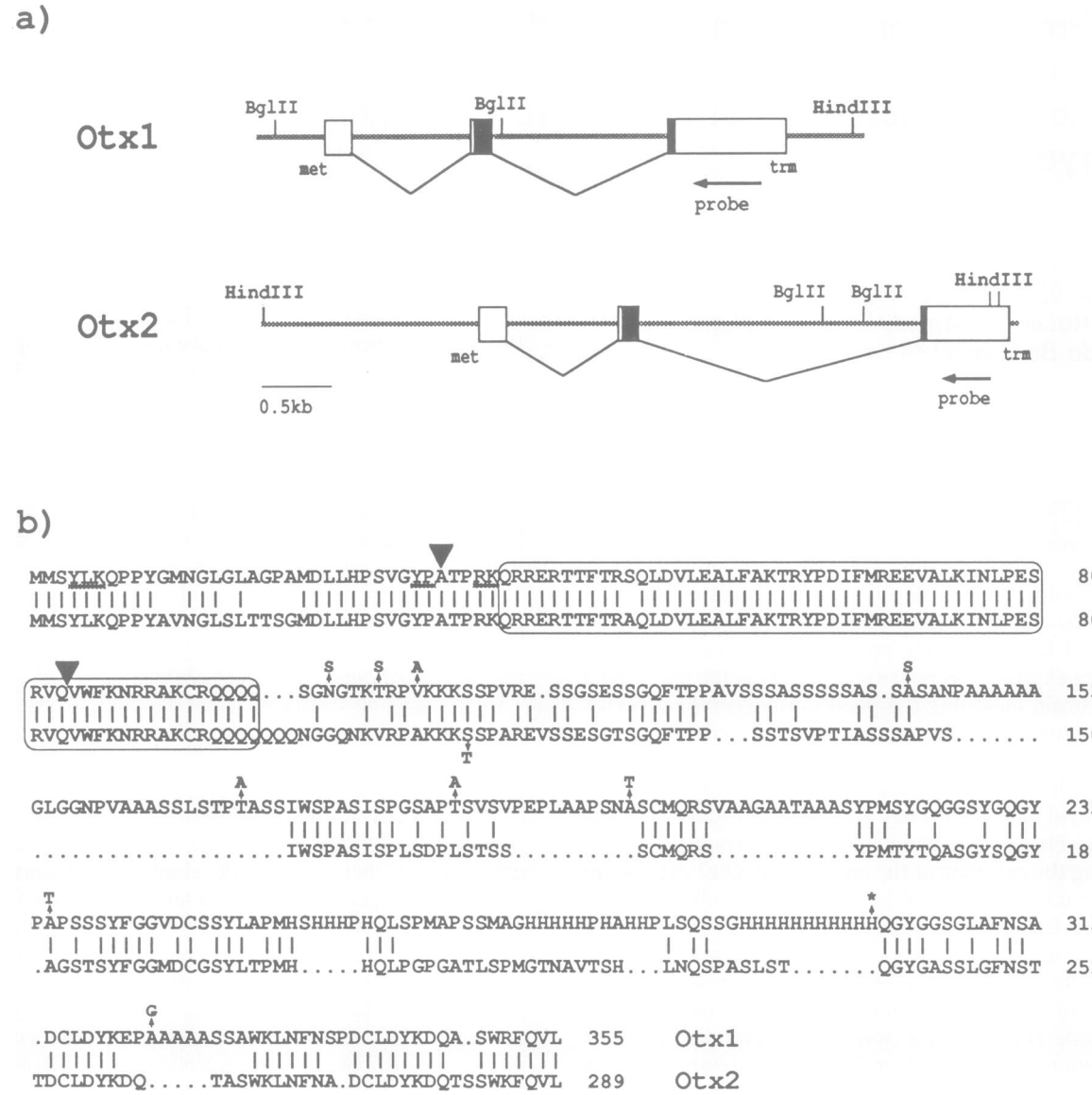

Fig. 1. Structure of *Otx1* and *Otx2*. (**a**) Genomic organization of *Otx1* and *Otx2*. Black boxes indicate the homeodomain. Probes used in Northern blot and *in situ* hybridization experiments are shown below the exon−intron scheme. (**b**) Deduced peptide sequence of human and mouse gene products. The murine *Otx1* and *Otx2* sequences are shown in full using the one-letter amino acid code and aligned. Single letters above and below the sequence indicate amino acid differences present in the human homologues; an asterisk indicates a deletion. Note that the human OTX2 and mouse Otx2 proteins differ by a single substitution, namely threonine to serine, out of 289 amino acid residues. The homeodomain is boxed. Additional peptide motifs conserved in the *Drosophila otd* gene product (Simeone *et al.*, 1992a) are underlined and arrowheads point to splice sites. (Accession numbers: P80205, P80206).

the onset of gastrulation. In midstreak embryos its expression appears progressively restricted to the anterior embryonic ectoderm corresponding to presumptive fore- and mid-brain. *Otx2* is one of the earliest genes known to be expressed in anterior neuroectoderm, demarcating rostral brain regions. Its gene product contains a homeodomain of the *bicoid* class and is able to recognize and transactivate a *bicoid* target sequence.

Results

Structure and expression of Otx1 and Otx2

The exon−intron organization and the peptide sequence of *Otx1* and *Otx2* are shown in Figure 1. The structure of the mouse *Otx1* and *Otx2* genes is shown in Figure 1a. The predicted peptide sequences of mouse and human *Otx1* and *Otx2* gene products are shown in Figure 1b. The proteins are 355 and 289 residues long, respectively. The two proteins are very similar to each other for the first 98 residues including the amino-terminal region, 37 residues upstream of the homeodomain and the homeodomain itself. Other regions of similarity are found downstream from the homeodomain. In Otx1 these regions are separated by stretches of additional residues including repetitions of alanine and histidine residues. The human OTX1 protein differs from its mouse homologue by nine substitutions and one deletion, whereas human OTX2 differs from the mouse homologue by a single conservative substitution, namely serine to threonine.

The Otx1 and Otx2 homeodomains differ from the Otd homeodomain by three and two amino acid residues respectively (Simeone *et al.*, 1992a). Notably the three homeodomains share the lysine residue at position 9 of the third, recognition, helix also present in the *Drosophila bicoid*

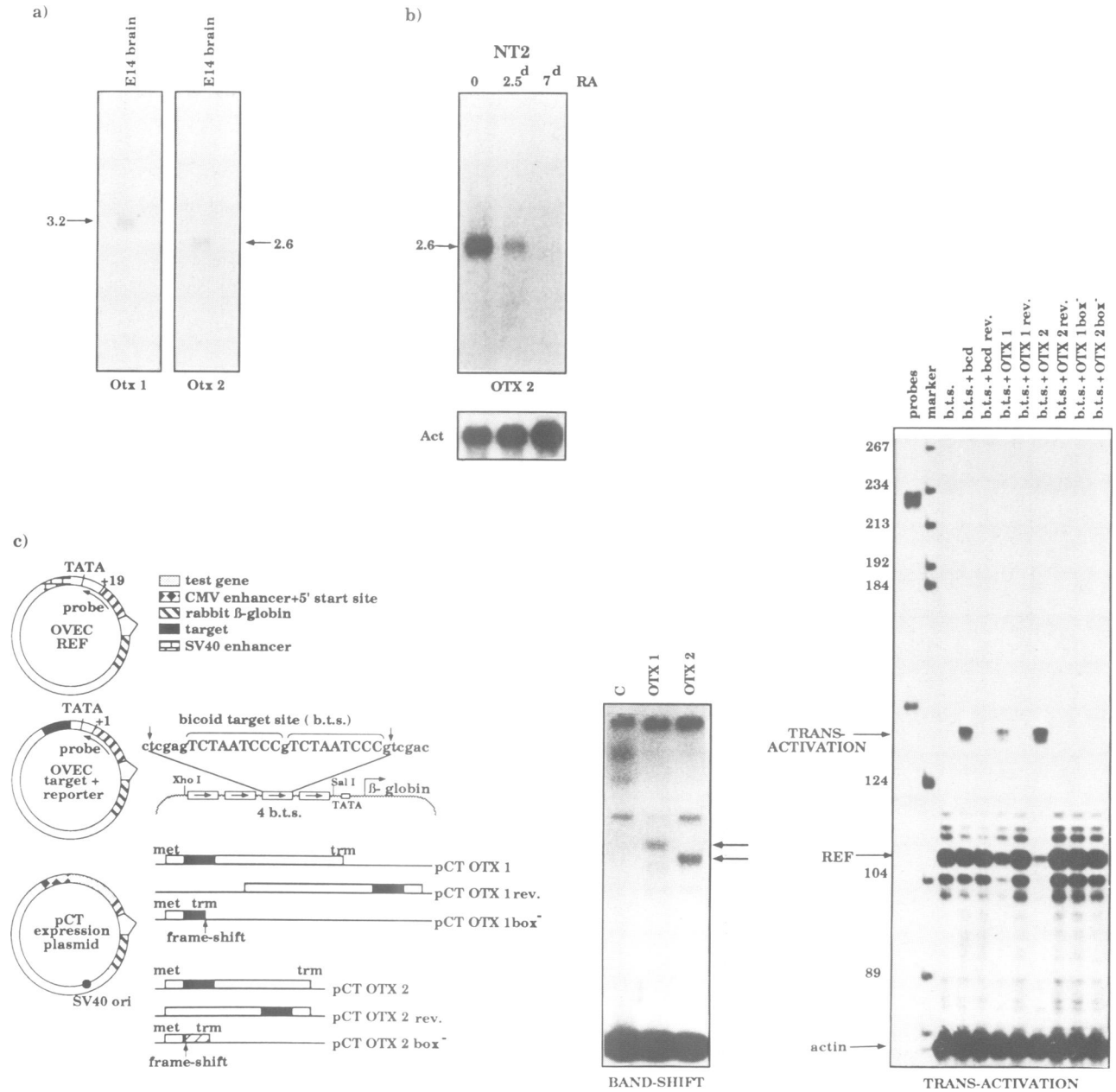

Fig. 2. Characterization of *Otx1* and *Otx2* gene products. (**a**) Expression of murine *Otx1* and *Otx2* in dissected brain of E14 mouse embryos. 3 μg of polyadenylated RNA per lane were analyzed by Northern blotting. Approximate transcript sizes are shown in kb. (**b**) Expression of the human *OTX2* gene in human embryonal carcinoma NTera2/clone D1 (NT2) cells, untreated and after 2.5 and 7 days of 10 μM retinoic acid treatment (RA). (**c**) DNA binding properties of the human OTX1 and OTX2 proteins. In the mobility shift experiments an oligonucleotide corresponding to the bicoid target site (b.t.s.) present in the transcriptional control region of *Drosophila hunchback* (Driever and Nüsslein-Volhard, 1989) gene was challenged with nuclear extracts from HeLa cells transfected with appropriate constructs expressing OTX1 and OTX2. Constructs are shown on the left hand side as pCT expression plasmids. A DNA binding activity is already present in nontransfected control (C) HeLa cells. On the right hand side an RNase protection analysis is shown of transactivation experiments. HeLa cells were transfected with a variety of pCT (Thali *et al.*, 1988) expression plasmids expressing different versions of Bcd, OTX1 and OTX2, in correct and reverse (rev.) orientation. box$^-$ indicates frameshift mutants yielding prematurely terminated products. These cells are also transfected with a reporter construct expressing rabbit β-globin under the control of four copies of the bts sequence in tandem. Expression of this reporter gene was detected and quantified by RNase protection of the indicated probe as was the expression of a reference OVEC plasmid (REF) (Thali *et al.*, 1988) and of endogenous β-actin. OVEC plasmid constitutively expresses a slightly shorter version of rabbit β-globin under the control of the SV40 enhancer sequence.

(*bcd*) (Driever and Nüsslein-Volhard, 1988) and frog *goosecoid* (Blumberg *et al.*, 1991) gene products. OTX1 and OTX2 also share with Otd two residues, i.e. Arg-Lys, immediately upstream from the homeodomain and a tripeptide, Tyr-Leu-Lys, at the amino-terminus. The three proteins also have the same general structure: all three contain a relatively short amino-terminal domain upstream from the homeodomain and a long carboxy-terminus downstream from it. An intron is present in *Otx1* and *Otx2* immediately upstream from the homeodomain, as is often the case for homeobox genes (Boncinelli *et al.*, 1991). The exon upstream from this intron ends in all three genes with the motif Tyr-Pro-Ala/Gly, possibly a divergent version of the conserved homeopentapeptide Ile/Phe-Tyr-Pro-Trp-Met

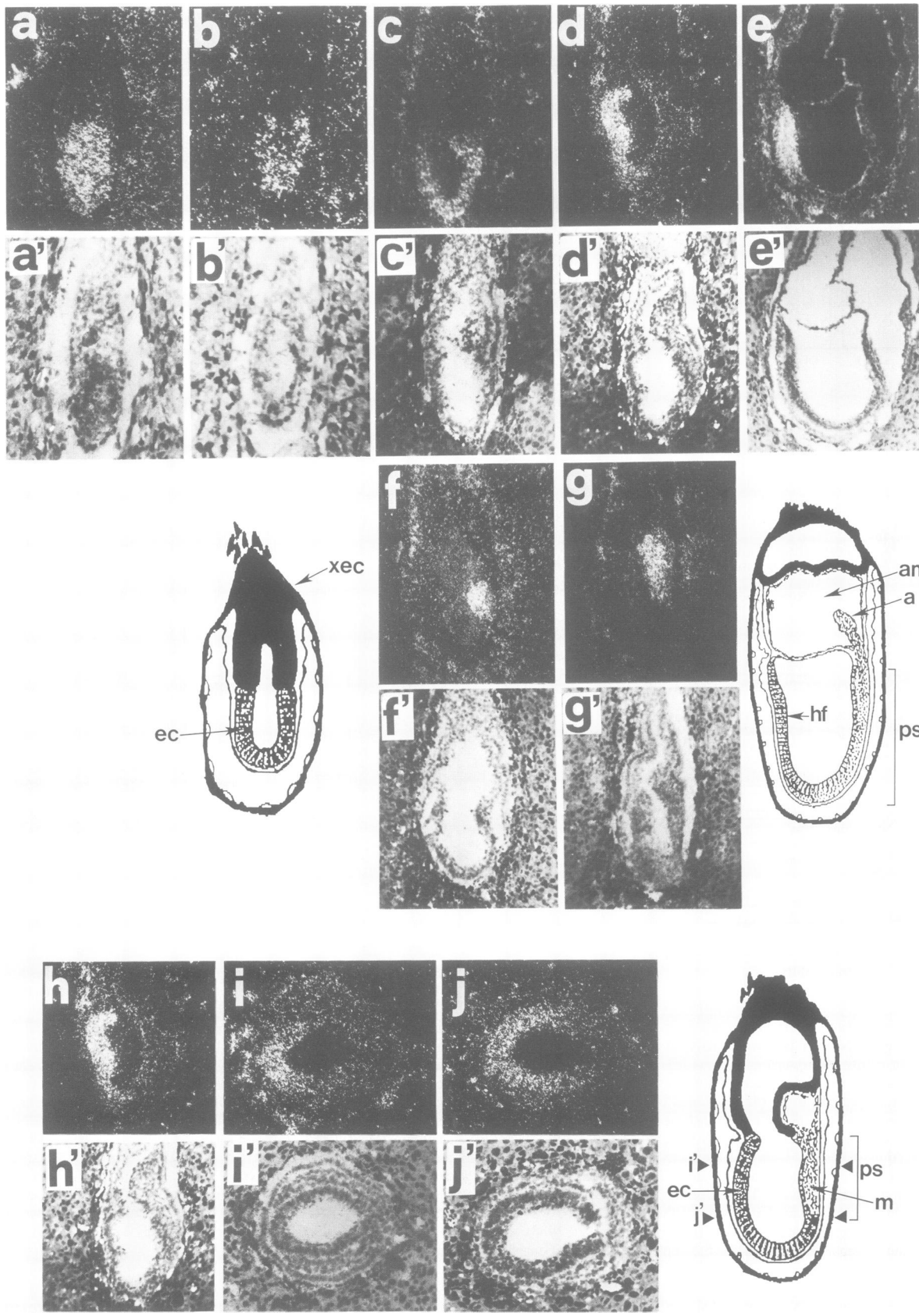

Fig. 3. Otx2 expression in sagittal (**a**–**h**) and transverse (**i**–**j**) sections of early mouse embryos. A bright-field image exactly corresponding to a given dark-field one is indicated by a prime suffix. Approximate ages in days post-coitum are 5.75 (a), 6.2 (b), 6.75 (c and f), 7 (d, g and h–j), 7.5 (e). Panels a–e and h–j show hybridization with *Otx2*; panels f and g show hybridization with murine *Evx1* (Bastian and Gruss, 1990; Dush and Martin, 1992). (h) is identical to (d) and serves as a reference for the corresponding transverse sections (i and j). Schemes of selected sections are also shown. a, allantois; am, amniotic cavity; ec, embryonic ectoderm or epiblast; hf, headfold; m, mesoderm; ps, primitive streak; xec, extraembryonic ectoderm.

present in several homeotic genes of *Drosophila* and in most vertebrate genes belonging to the Hox clusters (Boncinelli *et al.*, 1991). An additional intron is present in the three genes within the homeobox at identical positions, namely between residues 46 and 47 of the homeodomain.

Both genes are expressed in the head of E14 mouse embryos (Figure 2a) with mature mRNAs 3.2 and 2.6 kb long, respectively. *OTX1*, the human homologue of *Otx1*, is not expressed in human NT2/D1 embryonal carcinoma (EC) cells (Simeone *et al.*, 1991), whereas *OTX2* is expressed in these cells (Figure 2b). Its expression appears relatively abundant in EC stem cells prior to any treatment and slowly decreases after retinoic acid treatment. This is in contrast with what is observed in the same cells for homeobox genes of the Hox family (Boncinelli *et al.*, 1991) and the two *Emx* genes studied (Simeone *et al.*, 1992b). In fact, most Hox genes are activated in these cells upon retinoic acid treatment, whereas the two *Emx* genes appear to be silent in these cells even after retinoic acid addition.

Recognition of the bicoid target sequence

The presence of a *bcd*-like homeodomain in these genes suggests the possibility that their gene products recognize the *bcd* target sequence (bts) present in the transcriptional control region of *hunchback* (Driever and Nüsslein-Volhard, 1989). In order to test this hypothesis, we first studied the DNA binding properties of the human OTX1 and OTX2 proteins in an electrophoretic mobility shift assay (Figure 2c). An oligonucleotide (5′-TCTAATCCC-3′) corresponding to bts was challenged with nuclear extracts from HeLa cells transfected with appropriate constructs expressing OTX1 and OTX2. Both extracts are able to shift the oligonucleotide band in a specific manner even if a DNA binding activity appears to be already present in nontransfected HeLa cells.

We further tested the ability of the two proteins to transactivate a reporter gene controlled by the bts sequence in co-transfection experiments (Figure 2c). HeLa cells were transfected with a variety of pCT (Thali *et al.*, 1988) expression plasmids expressing different versions of the *bicoid*, *OTX1* and *OTX2* gene products. These cells were also transfected with a reporter construct expressing rabbit β-globin under the control of four tandem copies of the bts sequence. Expression of this reporter gene is detected and quantified by RNase protection as is the expression of a reference OVEC plasmid (Thali *et al.*, 1988), constitutively expressing a modified version of rabbit β-globin under the control of the SV40 enhancer sequence. All three proteins appear to activate the reporter construct with *OTX2* gene products activating more effectively than those of *bcd* and *OTX1*. *OTX1* and *OTX2* frameshift mutants yielding prematurely terminated products are unable to transactivate the reporter construct. In conclusion, the homeoproteins encoded by both *Otx* genes are able to recognize and bind the natural target sequence of *bicoid*, suggesting that the three proteins can bind similar DNA target sequences.

Otx2 early expression

The fact that *Otx2* is expressed in EC stem cells prompted us to investigate how early it is expressed in embryogenesis. We previously reported that it is already expressed in E7.5 embryos in an anterior neuroectodermal region of the embryo corresponding to the headfold (Simeone *et al.*, 1992a). *In situ* hybridization experiments (Figure 3) show that *Otx2* transcripts are detectable in embryonic structures from the prestreak stage onwards. *Otx2* is faintly expressed at about day 5.5−5.7 post-coitum in the embryonic ectoderm, also called primary ectoderm or epiblast (Figure 3a). The proximal boundary of its expression domain coincides with the border between extraembryonic and embryonic ectoderm. Essentially the same pattern is observed in E6.0−6.2 embryos when the proamniotic cavity becomes apparent (Figure 3b). At this stage the homeobox gene *Evx1* (Bastian and Gruss, 1990; Dush and Martin, 1992) is not yet expressed. It begins to be detectable just before the start of streak formation (Dush and Martin, 1992). In E6.5 embryos *Evx1* expression (Figure 3f) is clearly detectable in cells in the primitive streak and in lateral ectodermal cells near the embryonic−extraembryonic junction. In these embryos *Otx2* expression is still extended to the entire epiblast (Figure 3c), whereas between day 7 and 7.5 its expression will recede and become restricted to anterior regions. In midstreak embryos *Evx1* is expressed in cells within the primitive streak which has now extended to a point near the distal tip of the embryo. Its transcripts (Figure 3g) are detected in a somewhat graded fashion, with the highest levels in the proximal region of the primitive streak up to two-thirds of the distance from the embryonic−extra-embryonic border to the distal tip of the embryo. In these embryos Otx2 expression is confined to regions in the anterior third of the embryo (Figure 3d and h−j). In transverse sections (Figure 3i−j) its expression is clearly excluded from lateral mesoderm and posterior and lateral ectoderm. The *Otx2* expression pattern in late-streak embryos (Figure 3e) clearly involves only anterior regions of the embryo corresponding to part of the headfold, as previously reported (Simeone *et al.*, 1992a).

In conclusion, *Otx2* is first expressed in prestreak embryos in the entire epiblast, where it persists for hours after the onset of gastrulation. In midstreak embryos its expression is progressively restricted to the anterior embryonic ectoderm corresponding to presumptive fore- and mid-brain, in keeping with what we observed in later stages of development.

Expression domains in E12.5 embryos

Otx1. We previously reported (Simeone *et al.*, 1992a) the temporal profile of *Otx1* expression in E8−E10.25 embryos. *Otx1* is first expressed in a large region of the anterior neural tube of E8.5 embryos. Anterior−posterior delimitation of *Otx1* expression in the rostral neural tube is clear in E9−E10 embryos. Dorsally, its expression domain is comprised of a continuous region including part of the telencephalon, the diencephalon and the mesencephalon. The posterior boundary of this domain coincides with that of the mesencephalon. Ventrally, the *Otx1* expression domain includes contiguous regions of both diencephalon and mesencephalon with sharp anterior and posterior boundaries. Starting from 10.25 days of development Otx1 expression extends posteriorly to regions of metencephalon.

We have now studied its expression in E12.5 mouse embryos in sagittal (Figure 4) and frontal (Figure 5) sections. *Otx1* appears to be expressed in several regions of the head. In particular, in the brain it is expressed in telencephalon, diencephalon, mesencephalon and limited regions of metencephalon, but not in myelencephalon or spinal cord. In sagittal sections (Figure 4a−c), *Otx1* expression is detectable in the presumptive cerebral cortex from its anterior

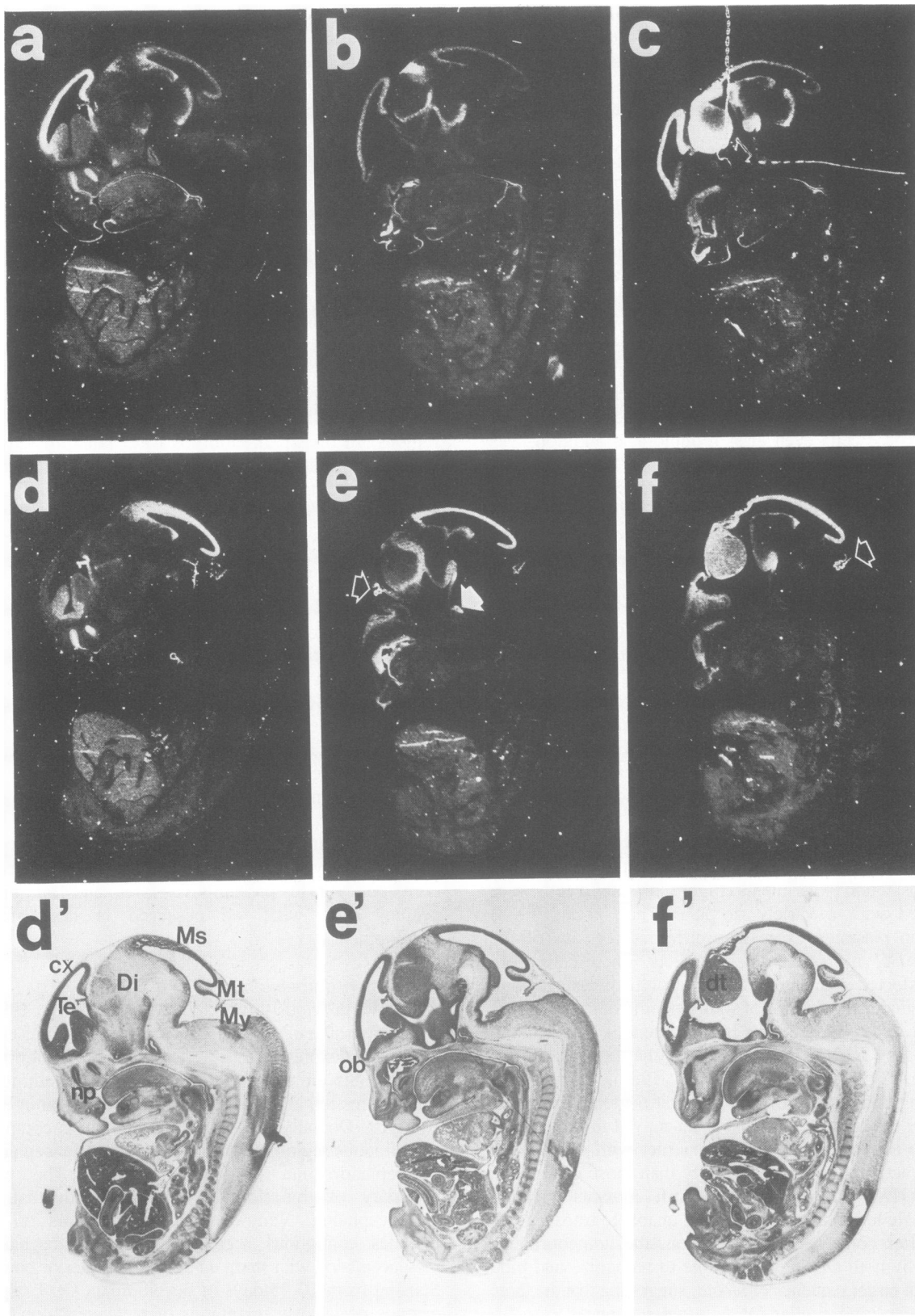

Fig. 4. *Otx1* and *Otx2* expression in sagittal sections of E12.5 mouse embryos. Panels **a**–**c** show hybridization with *Otx1*; panels **d**–**f** show hybridization with *Otx2*. Sections b and e are medial. Arrowheads in e and f point to choroid plexuses. A solid arrowhead in e points to neurohypophysis. cx, presumptive cortex; Di, diencephalon; dt, dorsal thalamus; Ms, mesencephalon; Mt, metencephalon; My, myelencephalon; np, nasal pits; ob, primordium of olfactory bulb; Te, telencephalon.

boundary to its posterior boundary. Hybridization signal is uniformly distributed across the cortex, without major differences. Sagittal sections in the middle of lateral ventricles (Figure 4b and c) reveal expression in the olfactory bulbs. *Otx1* expression is detectable in some noncortical basal telencephalic regions, namely in the germinal layer of the

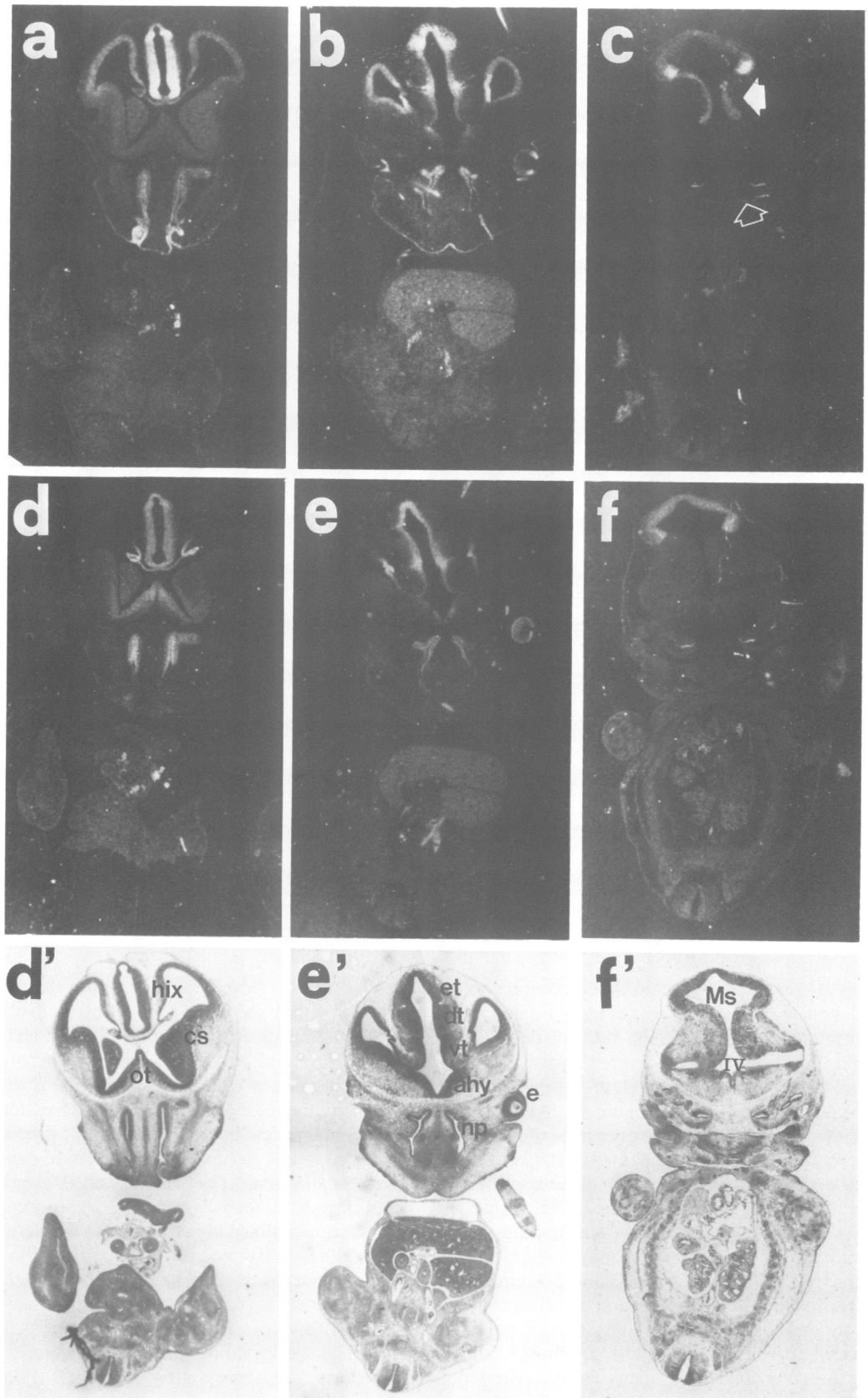

Fig. 5. *Otx1* and *Otx2* expression in frontal sections of E12.5 mouse embryos. Panels **a**–**c** show hybridization with *Otx1*. Panels **d**–**f** show hybridization with *Otx2*. An arrowhead in c points to the pharynx. A solid arrowhead in c indicates expression in the metencephalon. ahy, anterior hypothalamus; cs, corpus striatum; dt, dorsal thalamus; e, eye; et, epithalamus; hix, primordium of hippocampal cortex; Ms, mesencephalon; np, nasal pits; ot, olfactory tubercle; vt, ventral thalamus; IV, fourth ventricle.

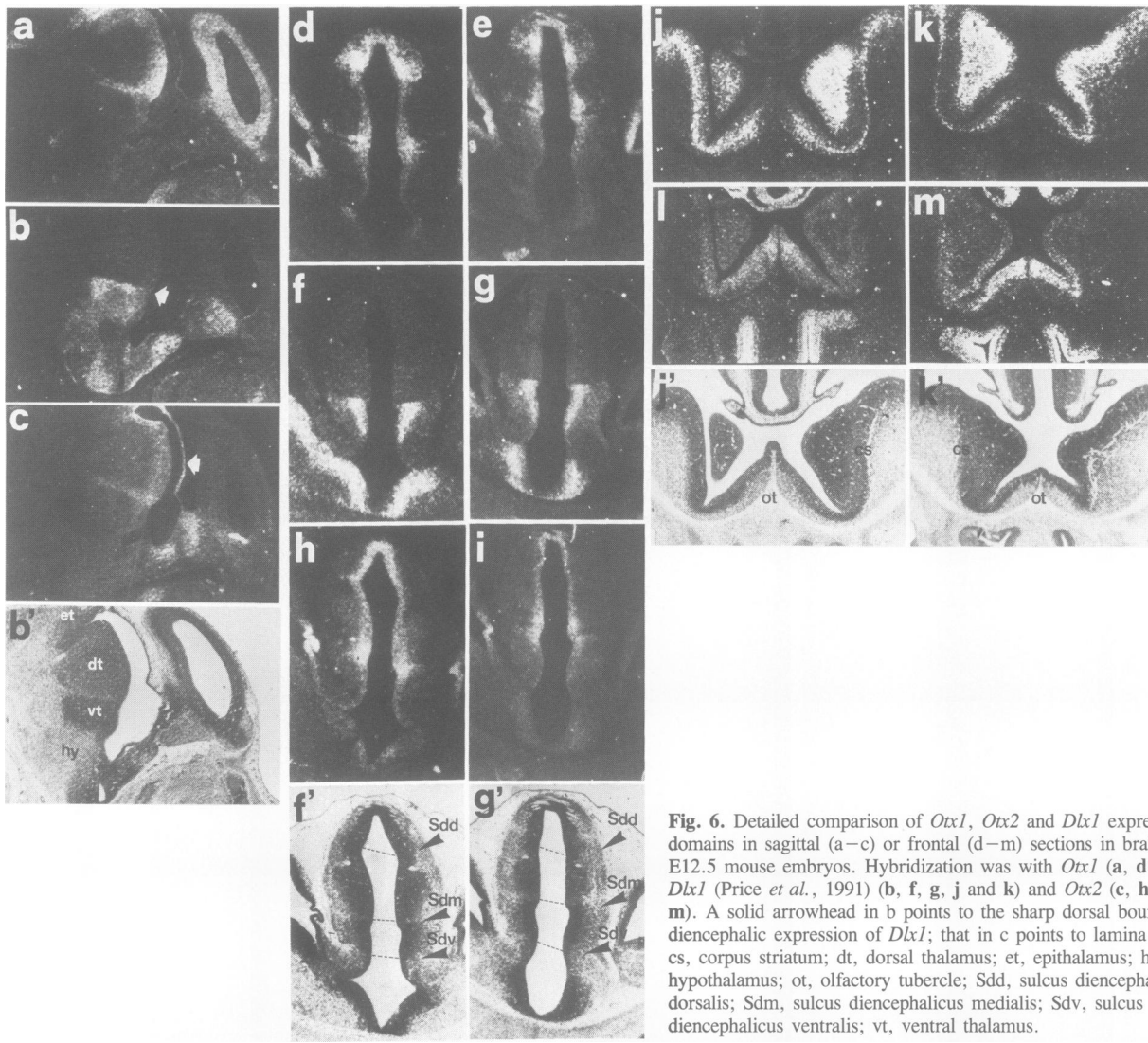

Fig. 6. Detailed comparison of *Otx1*, *Otx2* and *Dlx1* expression domains in sagittal (a−c) or frontal (d−m) sections in brain regions of E12.5 mouse embryos. Hybridization was with *Otx1* (**a**, **d** and **e**), *Dlx1* (Price *et al.*, 1991) (**b**, **f**, **g**, **j** and **k**) and *Otx2* (**c**, **h**, **i**, **l** and **m**). A solid arrowhead in b points to the sharp dorsal boundary in diencephalic expression of *Dlx1*; that in c points to lamina terminalis. cs, corpus striatum; dt, dorsal thalamus; et, epithalamus; hy, hypothalamus; ot, olfactory tubercle; Sdd, sulcus diencephalicus dorsalis; Sdm, sulcus diencephalicus medialis; Sdv, sulcus diencephalicus ventralis; vt, ventral thalamus.

most lateral portion of lateral ganglionic eminence (Figure 4a). It is also expressed in regions of diencephalon: epithalamus, dorsal thalamus and mammillary region of posterior hypothalamus. Its expression domain does not include the ventral thalamus. A two-layered narrow stripe of expression is detectable at the level of the boundary between dorsal and ventral thalamus (Figure 4b and c), that is the zona limitans intrathalamica, the precursor of lamina medullaris externa and mammillo-thalamic tract (see also Figure 6a, d and e). There is also expression in the fasciculus retroflexus, the precursor of habenulo-interpenduncular tract, stria medullaris, including the region surrounding the posterior commissure, primordium of mammillotegmental tract, fornix and sulcus lateralis hypothalami posterioris. Posterior to diencephalon, it is expressed in mesencephalic regions of tectum and tegmentum, possibly at the level of presumptive dorsal periventricular bundle (Figures 4c and 5c). The *Otx1* expression domain maintains a sharp posterior boundary, both dorsally (Figure 4a−c) and ventrally (Figure 4a and c) at the level of the rhombic isthmus, already shown in earlier stages (Simeone *et al.*, 1992a). Ventrally, however, its expression reappears, after a gap just posterior to the 4th cranial nerve, in the anterior metencephalon, the presumptive pontine region.

Otx1 is expressed not only in the developing brain, but also in epithelia in the nasal cavities (Figure 4a−c), in the cochlear duct of developing ear and in the developing eye (not shown but see Figure 7). *Otx1* is also expressed in ectodermal regions in the snout (Figure 4a and b) and in the lining of the oral cavity including the thyro-glossal duct.

The extension of *Otx1* expression in the head is confirmed in frontal sections (Figure 5). Its expression in cortical telencephalon includes neopallium, hippocampal and parahippocampal archipallium and selected palaeopallial and septal localizations. *Otx1* expression is also confirmed in noncortical basal telencephalic regions, especially in part of the superior basimedial region and in the germinal layer of lateral ganglionic eminence. It is expressed in the germinal layer of diencephalon, particularly at the level of two sulci, namely the diencephalicus dorsalis and medius (see also Figure 6d and e). Additional localizations are posterior hypothalamus including the sulcus lateralis hypothalami posterioris, mesencephalon (Figure 5c) and anterior metencephalon (Figure 5c, solid arrowhead). Noncerebral localizations are in ocular regions (Figure 5b), nasal cavities (Figure 5a,b) including external ducts (Figure 5a) and pharynx (Figure 5c, arrowhead).

Otx2. Let us now consider *Otx2* expression in E12.5 mouse embryos. We previously reported (Simeone *et al.*, 1992a) the temporal profile of *Otx2* expression in E7.5−E10.25

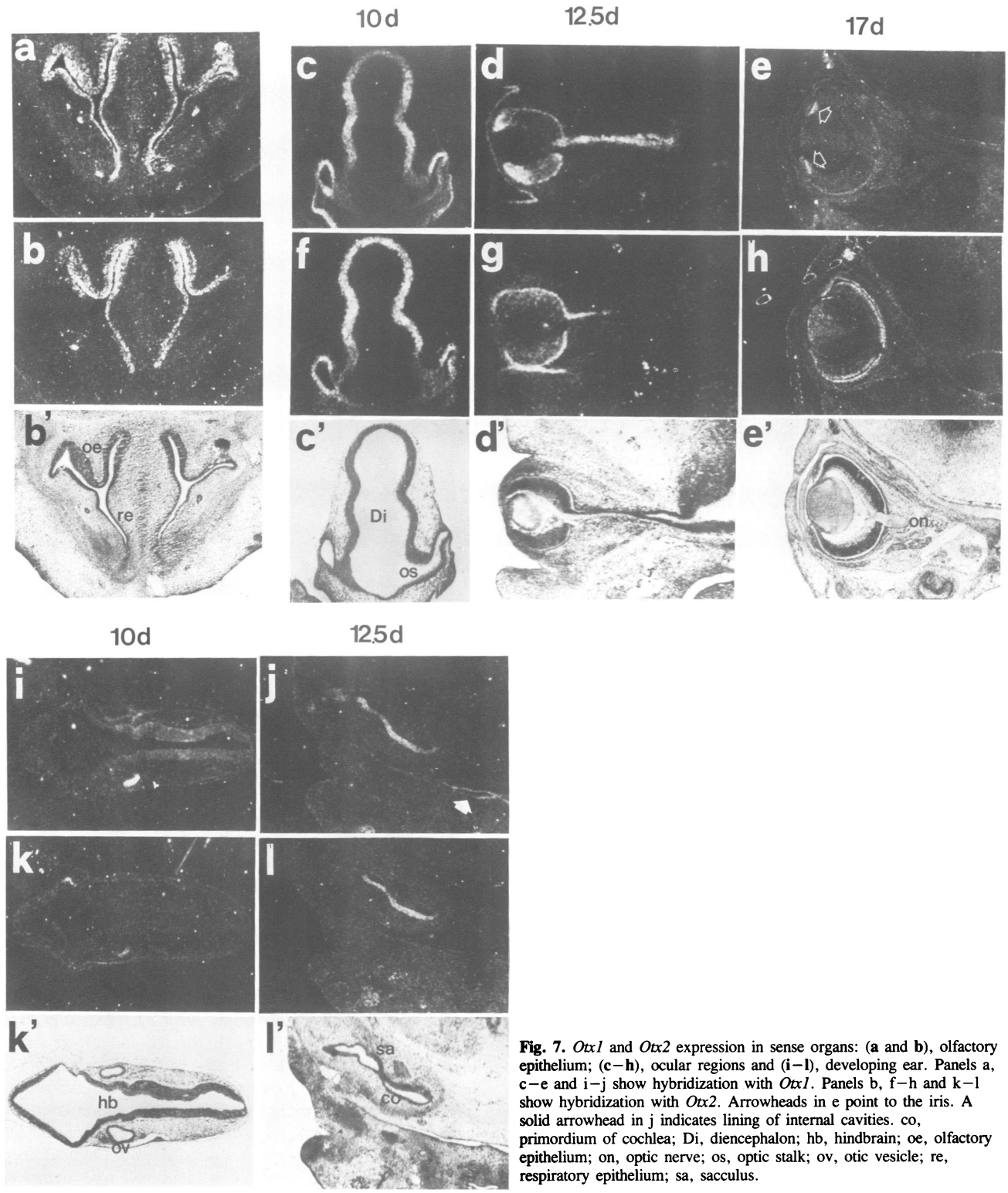

Fig. 7. *Otx1* and *Otx2* expression in sense organs: (**a** and **b**), olfactory epithelium; (**c**–**h**), ocular regions and (**i**–**l**), developing ear. Panels a, c–e and i–j show hybridization with *Otx1*. Panels b, f–h and k–l show hybridization with *Otx2*. Arrowheads in e point to the iris. A solid arrowhead in j indicates lining of internal cavities. co, primordium of cochlea; Di, diencephalon; hb, hindbrain; oe, olfactory epithelium; on, optic nerve; os, optic stalk; ov, otic vesicle; re, respiratory epithelium; sa, sacculus.

embryos. As already mentioned, *Otx2* is expressed in very anterior regions of E7.5 embryos including the headfold. *Otx2* is also expressed in E8.5–E10 embryos with an expression domain containing, both dorsally and ventrally, that of *Otx1* and basically including the entire forebrain. Dorsally, it includes the entire telencephalon, the diencephalon and the mesencephalon. Ventrally, the *Otx2* expression domain includes contiguous regions of diencephalon and mesencephalon with an anterior boundary just posterior to the optic chiasma. Starting from day 11.75 post-coitum, *Otx2* expression disappears from dorsal telencephalon.

Sagittal (Figure 4) and frontal (Figure 5) sections of E12.5 embryos reveal that *Otx2* is not expressed at this stage in cortical telencephalon (see also Figure 6c) but in a subset of presumptive noncortical basal ganglia. Like *Otx1*, *Otx2* appears to be expressed in several regions of the head. In particular, in the brain it is expressed in diencephalon and mesencephalon but not in metencephalon, myelencephalon or spinal cord. In sagittal sections (Figure 4d–f), *Otx2*

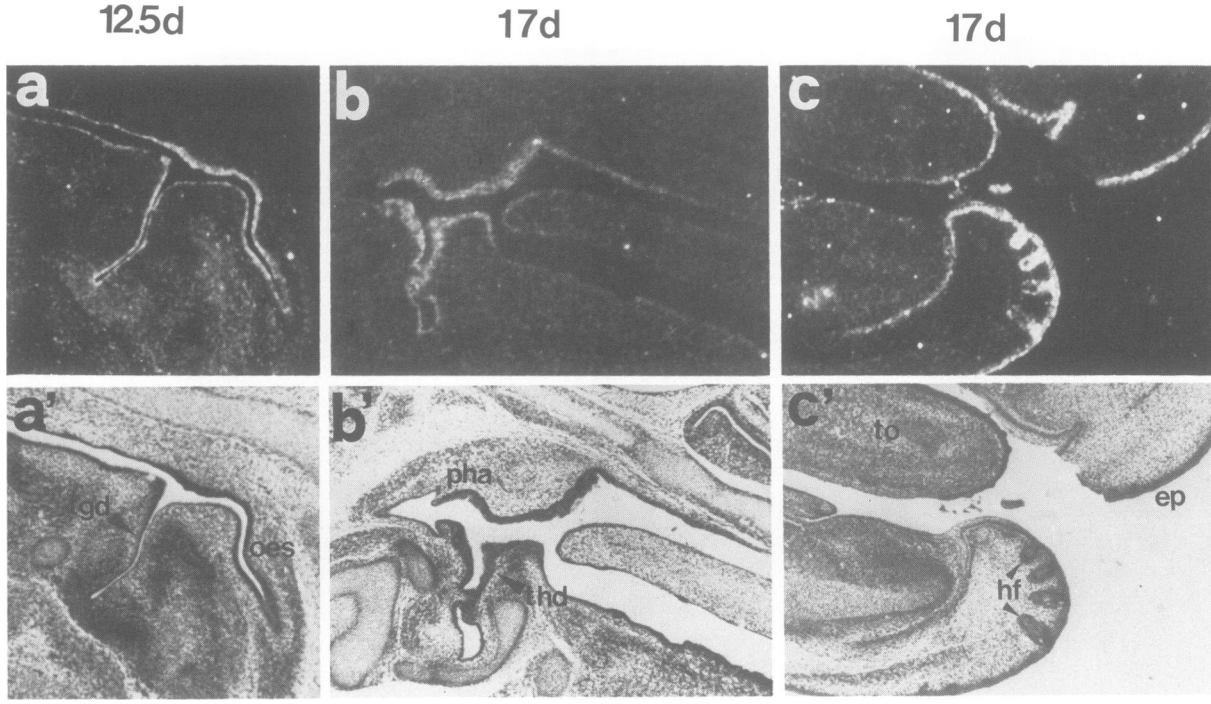

Fig. 8. Additional localizations of *Otx1* expression: (**a** and **b**) lining of internal cavities; (**c**) lower jaw. ep, epithelium; hf, hair follicles; pha, pharynx; oes, oesophagus; tgd, thyro-glossal duct; thd, thyroid duct; to, tongue.

expression is detectable in noncortical basal telencephalic regions. It is not expressed in the anterior septal region but is very strongly expressed in septal regions contiguous to diencephalon and in the germinal layer of anterior basi-medial regions (Figure 5d). Its expression in lamina terminalis (see also Figure 6c) confirms its very anterior expression already shown earlier in development (Simeone *et al.*, 1992a). This is also in keeping with its expression in medial septal regions. *Otx2* is expressed in regions of diencephalon and mesencephalon with a pattern very similar to *Otx1* (Figure 4d–f and 5d–f). *Otx2* is clearly expressed in the anlage of neurohypophysis (Figure 4e, solid arrowhead) and in choroid plexuses in outer ventriculi (Figure 4e, arrowhead and Figure 5d and e) and myelencephalon (Figure 4f, arrowhead). *Otx2* is not only expressed in the developing brain: its expression domain in the head appears to extend to epithelia in the nasal cavities (Figures 4d–f and 5d and e) and to developing ear and eye (Figure 5e).

Expression of both *Otx* genes in the developing brain remains essentially the same up to E15 embryos and progressively declines later on (not shown).

Comparison with Dlx1 expression

Dlx1 (Price *et al.*, 1991) is a homeobox gene, related to *Drosophila Distal-less*, that has been reported to be expressed in ventral thalamus and basal telencephalic regions. We hybridized *Dlx1* on contiguous sections in order to compare its expression pattern with those of *Otx1* and *Otx2*. There is a certain degree of complementarity between the localization of *Dlx1* expression and that of the *Otx* genes both in diencephalon, striatum and basi-medial region. This comparison is shown in detail in Figure 6. In sagittal sections of diencephalon (Figure 6a–c) the dorsal border (Figure 6b, solid arrowhead) of *Dlx1* expression abuts the ventral border of *Otx* expression (Figure 6a and c). This comprises two layers of cells orthogonal to the frontal surface of diencephalon and particularly evident for *Otx1* in Figure 6a, surrounding the zona limitans intrathalamica present at the boundary between dorsal and ventral thalamus. *Otx* genes appear in general to be expressed in germinal layers and boundary regions whereas *Dlx1* expression extends to more internal regions, such as paraventricular and internal areas of ventral thalamus (Figure 6b). Much the same is observable in frontal sections of diencephalon (Figure 6d–i) where the four longitudinal columns of Herrick, i.e. epithalamus, dorsal and ventral thalamus and hypothalamus, are easily identifiable (broken lines in Figure 6f′ and g′), being separated by sulci termed (from dorsal to ventral) sulcus diencephalicus dorsalis, medius and ventralis. *Otx1* (Figure 6d and e) and *Otx2* (Figure 6h and i) are expressed in ependymal layers of epithalamus and dorsal thalamus and in orthogonal layers of cells immediately above and below the sulcus diencephalicus medius. *Dlx1* is expressed in deeper regions of ventral thalamus (Figure 6f and g), as already reported (Price *et al.*, 1991, 1992). Complementarity of *Otx2* and *Dlx1* expression patterns is apparent in frontal sections of anterior basal regions (Figure 6j–m) where again *Otx2* expression (Figure 6l and m) is more superficial and *Dlx1* expression (Figure 6j and k) is distributed throughout deeper regions suggesting that these two genes have roles in different groups of neuroblasts.

Sense organs

Details of *Otx1* and *Otx2* expression in developing sense organs are shown in Figure 7. In nasal cavities of E12.5 embryos, both *Otx1* (Figure 7a) and *Otx2* (Figure 7b) are expressed in olfactory epithelium as well as in the respiratory epithelium but with different patterns: *Otx2* is not expressed in the lateral wall of respiratory pits whereas *Otx1* is expressed in both the lateral and medial walls.

In the developing eye (Figure 7c–h) *Otx1* and *Otx2* are

both expressed but with different patterns. They are already expressed in optic stalks of E10 embryos (Figure 7c and f). In E12.5 embryos *Otx1* is expressed in the iris, in a peripheral region including ciliary bodies, sclera, external ectoderm and the external sheath of the optic nerve (Figure 7d), whereas in E17 embryos it is expressed in the iris (Figure 7e, arrowheads). In E12.5 embryos *Otx2* is expressed in sclera, retinal pigmented layer and leptomeninges intimately surrounding optic nerve (Figure 7g), whereas in E17 embryos it is expressed in the pigmented epithelium of retina and in neurosensory retina (Figure 7h).

Otx1 and *Otx2* are also expressed in developing ear (Figure 7i–l) from early expression in the otic vesicle (Figure 7i and k) to epithelia in auricular ducts of sacculus and cochlea.

Details of additional localizations of *Otx1* expression are shown in Figure 8. Expression is clearly detected in the lining of upper oral cavities (Figure 8a and b), including mucociliary epithelium of pharynx, oesophagus and thyroglossal duct. *Otx1* is also expressed in hair follicles in the jaw (Figure 8c) and epithelia of bronchioli (not shown).

Discussion

Otx2 in early embryogenesis

We report the identification and characterization of two mouse genes, *Otx1* and *Otx2*, containing a homeodomain homologous to that of *otd* (Finkelstein *et al.*, 1990), a *Drosophila* gene playing a major role in the developing head. Both *Otx* genes are expressed in the developing brain and in a few additional cephalic localizations. *Otx2* expression is confined to the head, whereas *Otx1* is mainly but not exclusively expressed in the head. Similarly, *Emx1* expression (Simeone *et al.*, 1992b) is confined to the head. Conversely, the 38 homeobox genes of the Hox family are exclusively expressed in the trunk (Boncinelli *et al.*, 1991; McGinnis and Krumlauf, 1992).

Separation of the head and trunk structures occurs very early in the development of both insects and mammals. In flies a cephalic furrow forms in gastrulating embryos at the posterior boundary of presumptive head at about one-third of the embryo length under the genetic control of *bicoid* (Driever and Nüsslein-Volhard, 1988). In short germ-band insects, embryos show an early determination of only the anterior head segments, whereas the more posterior thoracic and abdominal segments are sequentially added after formation of a primary germ anlage. There is also increasing evidence that the rules governing head formation may differ from the paradigm established for the central region of the body (Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991). In the mouse embryo the head derives from regions anterior to the primitive streak whereas most of the trunk paraxial mesoderm derives from growing cells stemming from the most anterior portion of the developing primitive streak (Tam, 1989). The embryonic axis lengthens up to the neural plate stage essentially by two processes: elongation of the primitive streak and expansion of the region of epiblast immediately cranial to the anterior end of the primitive streak (Lawson *et al.*, 1991).

Otx2 transcripts are detectable in embryonic tissues from the prestreak stage onwards (Figure 3). In fact, *Otx2* is faintly expressed at about day 5.5–5.7 post-coitum in the embryonic ectoderm or epiblast, and not in extraembryonic ectoderm (Figure 3a). The proximal boundary of its expression domain coincides with the border between extraembryonic and embryonic ectoderm. The epiblast of the gastrulating mouse embryo is believed to be the sole source of all definitive tissues in the fetus (Tam, 1989; Lawson *et al.*, 1991). The same expression pattern is observed in E6 embryos when the proamniotic cavity becomes apparent (Figure 3b) and in early-streak E6.5 embryos. At this stage the homeobox gene *Evx1* (Bastian and Gruss, 1990; Dush and Martin, 1992) begins to be detectable in cells in the primitive streak and in lateral ectodermal cells near the embryonic–extraembryonic junction. Between day 7 and 7.5 Otx2 expression progressively recedes to anterior regions, where it will remain confined (Figure 3d, h–j). These regions correspond to neuroectoderm of prosencephalon and mesencephalon where *Otx2* will remain expressed until late in gestation.

The progressive confinement of *Otx2* expression from the entire epiblast to presumptive fore- and mid-brain neuroectoderm occurs concomitantly with progressive regionalization of cell fate within the epiblast. This *Otx2* progressive confinement must correlate with the expression of other early developmental genes. Our analysis shows that it is unlikely that *Evx1* plays a direct role in this process. Conversely, what is known about the evolution of expression patterns of early Hox genes, in particular *Hox-2.9* (Wilkinson *et al.*, 1989; Frohman *et al.*, 1990), could suggest a relationship between the progressive displacement towards the anterior of both the anterior border of the Hox expression domain and the posterior border of the *Otx2* expression domain. This relationship remains to be investigated. *Otx2* is also expressed in NT2 stem cells and is downregulated in differentiating NT2 cells after retinoic acid addition (Figure 2d). This observation suggests a similarity between NT2 stem cells and epiblast cells. This is in keeping with progressive Hox gene activation and with the finding that *Emx* genes are never expressed in these cells (Simeone *et al.*, 1992b).

The Otx1 and Otx2 homeodomains differ by three and two amino acid residues from the Otd homeodomain, respectively. In particular, the three homeodomains share the lysine residue at position 9 of the recognition helix also present in the *bcd* and *goosecoid* gene products. The substitution of this lysine by glutamine in the Bcd homeodomain has been shown to replace its DNA binding specificity with that of the Antennapedia-like homeoproteins (Hanes and Brent, 1989). Thus, the presence of a Bcd-like homeodomain suggests the possibility that the products of the *Otx1* and *Otx2* genes recognize *bcd* target sequences, for example those present in the transcriptional control region of *hunchback* (Driever and Nüsslein-Volhard, 1989). We tested the ability of the human OTX1 and OTX2 proteins to bind an oligonucleotide corresponding to the *bcd* target sequence and to transactivate a reporter gene controlled by the *bcd* target sequence in co-transfection experiments in HeLa cells. The *bcd*, *OTX1* and *OTX2* gene products appear to activate the reporter construct with OTX2 activating more effectively than Bcd and OTX1 (Figure 2c). In conclusion, the homeoproteins encoded by both *Otx* genes are able to recognize and bind the natural target sequence of the *bcd* gene product, suggesting that the three proteins can bind similar DNA target sequences. The similarity between the

homeodomains of Otx2 and Bcd might be significant, even if there are many obvious differences between the two genes. *Otx2* and *bcd* are both expressed in anterior regions of early embryos, in mice and flies respectively. These regions demarcate rostral portions of head and brain and transiently define the anterior polarity.

Otx gene expression in the developing head

Later in development the two *Otx* genes are expressed in specific regions of the brain (Figures 4 and 5). Both are expressed in basal telencephalon, diencephalon and mesencephalon but not in spinal cord of E12.5 embryos. *Otx1* is also expressed in dorsal telencephalon and regions of metencephalon, whereas *Otx2* expression is localized specifically in choroid plexuses. Both genes are expressed in various regions of diencephalon, namely epithalamus, dorsal thalamus and mammillary region of posterior hypothalamus. Their expression domain does not include the ventral thalamus. A two-layered narrow stripe of expression is detectable at the level of the boundary between dorsal and ventral thalamus (Figure 6a,d,e and c,h,i), that is the zona limitans intrathalamica, the precursor of lamina medullaris externa. Posterior to diencephalon, they are expressed in mesencephalon and from E9.5 onward the expression of both genes clearly marks the posterior dorsal boundary of mesencephalon to the exclusion of presumptive cerebellar areas (Simeone *et al.*, 1992a).

Expression of both genes along the zona limitans intrathalamica appears of particular interest. Their expression domains might constitute a framework for the axon patterning of lamina medullaris and other structures separating dorsal thalamus from ventral thalamus. On contiguous sections we hybridized *Dlx1*, a homeobox gene related to *Distal-less* (Price *et al.*, 1991, 1992). In sagittal (Figure 6a–c) and frontal (Figure 6d–i) sections of diencephalon the dorsal border of *Dlx1* expression abuts the ventral border of *Otx* expression. Expression of *Otx* genes in two layers of cells orthogonal to the frontal surface of diencephalon and surrounding the zona limitans intrathalamica present at the boundary between dorsal and ventral thalamus might also account for the sharp dorsal boundary of *Dlx1* expression domain in ventral thalamus.

There is complementarity between localizations of *Dlx1* expression and that of the *Otx* genes both in diencephalon and basi-medial regions of telencephalon. *Otx* genes appear in general to be expressed in germinal layers and boundary regions whereas *Dlx1* expression extends to more internal regions of ventral thalamus and anterior basal regions (Figure 6). The exclusion of cells expressing *Dlx1* from ventricular germinal layers suggest (Price *et al.*, 1992) that these cells represent early differentiated neurons. Alternatively, *Dlx1* expression might be transient in proliferating neuroblasts. *Dlx1* expression appears to respond to already established neuromeric boundaries. Conversely, the *Otx* genes could be involved in the early subdivision of the forebrain.

Sense organs

Otx genes are also expressed in developing sense organs (Figure 7). In nasal cavities of E12.5 embryos, both *Otx1* and *Otx2* are expressed in olfactory epithelium as well as in the respiratory epithelium. *Otx1* and *Otx2* are expressed with similar patterns in the developing ear (Figure 7i–l) from early expression in the otic vesicle to epithelia in auricular ducts of sacculus and cochlea. In the developing eye (Figure 7c–h) *Otx1* and *Otx2* are already expressed in optic stalks of E10 embryos. In E12.5 embryos *Otx1* is expressed in the iris, sclera, external ectoderm and the external sheath of the optic nerve, whereas in E17 embryos it is expressed in the iris. In E12.5 embryos *Otx2* is expressed in sclera, retinal pigmented layer and leptomeninges intimately surrounding optic nerve, whereas in E17 embryos it is expressed in the pigmented epithelium of retina and in neurosensory retina. Expression of both genes around the optic nerve appears again of particular interest and may be similar to that along the zona limitans intrathalamica in providing clues to axon pathfinding and patterning.

Lethal alleles of *otd* cause abnormalities in the embryonic head which reflect an early role in anterior pattern formation, particularly in the antennal and preantennal regions of the head (Wieschaus *et al.*, 1984, 1992; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). In addition to its early role as a putative gap gene in the anterior region of the embryo, *otd* is also required later on as homozygous *otd* embryos show abnormalities in the specification of medial cells in the central nervous system (Finkelstein *et al.*, 1990; Klämbt *et al.*, 1991). In this respect, it is interesting to note that *Otx2* is expressed in the midline of rostral brain from the very beginning of neurulation (Simeone *et al.*, 1992a). Finally, *otd* is required for the development of the eye–antennal imaginal discs. We may conclude that developmental genes encoding homeoproteins containing an Otd-like homeodomain play a major role in the development of sense organs from a very remote past in evolution. This role 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 *otd* genomic sequence including the homeobox (Finkelstein *et al.*, 1990). Two classes of homologous cDNA clones, termed *OTX1* and *OTX2*, 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.

Mice and cells

C57/Bl6 mice were mated between 21 00 and 10 00 h. Day 0.5 post-coitum was assumed to begin at the middle of the day of vaginal plugging. Staging of early embryos was mainly according to Dush and Martin (1992). Pregnant female mice were killed by cervical dislocation and embryos were collected in ice-cold PBS under a dissection microscope (Zeiss SV11), and fixed overnight in 4% paraformaldehyde. Human EC cells of the NTERA-2 line, clone D1, (NT2/D1) (Andrews, 1984) were cultured and treated as previously reported (Simeone *et al.*, 1991).

HeLa cells were transfected with a variety of pCT (Thali *et al.*, 1988) expression plasmids expressing different versions of the *bcd*, *OTX1* and *OTX2* gene products. These cells were also transfected with a reporter construct expressing rabbit β-globin under the control of four copies of the *bcd* target sequence (bts, 5′-TCTAATCCC-3′) in tandem. Expression of this reporter gene is detected and quantified by RNase protection as is the expression of a reference OVEC plasmid (Thali *et al.*, 1988), constitutively expressing a modified version of rabbit β-globin under the control of SV40 enhancer sequence. All three proteins appear to activate the reporter construct whereas *OTX1* and *OTX2* frameshift mutants yielding prematurely terminated products are unable to transactivate it.

Preparation of ^{32}P- and ^{35}S-labelled RNA probes for Otx1 and Otx2 for RNase protection and in situ hybridization

Otx1 and *Otx2* sense and antisense RNA probes were synthetically produced using respectively a 396 nucleotide *Sac*I–*Pst*I fragment and a 263 nucleotide

*Hae*III fragment as templates (Figure 1a). 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 the 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 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 nonspecifically 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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