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Xenopus Distal-less related homeobox genes are expressed in the developing forebrain and are induced by planar signals

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SUMMARY

The polymerase chain reaction (PCR) was used to isolated five *Xenopus* homeobox clones (*X-dll1* to *5*) that are related to the *Drosophila Distal-less* (*Dll*) gene and we propose a subdivision of the vertebrate *distal-less* gene family according to sequence similarities. cDNA clones were isolated for *X-dll2, 3* and *4,* and their expression was studied by RNase protection and in situ hybridization. *X-dll2,* which belongs to a separate subfamily than *X-dll3* and *4,* is not expressed in the neural ectoderm. *X-dll3* and *X-dll4,* which belong to the same subfamily, have a similar but not identical pattern of expression that is restricted to anterior ectodermal derivatives, namely the ventral forebrain, the cranial neural crest and the cement gland. *X-dll3* is also expressed in the olfactory and otic placodes while *X-dll4* is expressed in the developing eye. *X-dll3* differs from the other *Xenopus* genes and the previously isolated *Dll*-related mouse genes, in that localized expression can be detected by in situ hybridization very early in development, in the anterior-transverse ridge of the open neural plate. Based on that early expression pattern, we suggest that *X-dll3* marks the rostral-most part of the neural plate, which gives rise to the ventral forebrain. Finally, we have used these *Xenopus distal-less* genes to show that the anterior neural plate can be induced by signals that spread within the plane of neural ectoderm, indicating that at least the initial steps of forebrain development do not require signals from underlying mesoderm.

Key words: *Xenopus*, *distal-less*, homeobox, forebrain, sense organs, neural crest, planar induction

INTRODUCTION

The development of the vertebrate nervous system begins with the induction of the neural plate on the dorsal surface of the embryo near the completion of gastrulation. A process closely linked to neural induction is the regionalization of the neural ectoderm along the anteroposterior (A-P) axis, into prospective forebrain, midbrain, hindbrain and spinal cord. The neural plate can also be divided along the A-P axis according to the character of the underlying mesoderm (reviewed in Bergquist and Källén, 1954; Sáxen, 1989). The part of the nervous system posterior to, and including, the midbrain overlies the chordal mesoderm (notochord), while the forebrain is derived from the portion of the neural plate that overlies the prechordal mesoderm. There are several differences in the development of the chordal and prechordal mesoderm and the respective overlying parts of the nervous system. For example, neither the prechordal mesoderm nor the overlying forebrain undergo the powerful convergent-extension movements during gastrulation and neurulation that are characteristic of more posterior regions of the axis (Keller et al., 1992a, b). Later differences include the induction of a floor plate by the notochord in the epichordal neural ectoderm, which does not take place in the prechordal forebrain (van Straaten et al., 1989; Yamada et al., 1991; Placzek et al., 1991).

The mechanisms that establish A-P pattern within the neural ectoderm have been studied using molecular markers, most of which are homeobox-containing genes. Homeobox genes encode putative transcription factors characterized by the presence of a highly conserved 60 aminoacid (aa) DNA-binding motif. Based on sequence similarity, homeobox genes have been classified into several families that include genes from various vertebrates and from *Drosophila* (Scott et al., 1989). A family of vertebrate genes that show similarity to the *Drosophila* genes of the *ANT-C* and *BX-C* complexes, the *Hox* genes, have been strongly implicated in the A-P patterning of the axial skeleton and the CNS, based on the strong evolutionary conservation of sequence and gene structure, their expression pattern, and the outcome of overexpression and loss-of-function experiments (reviewed in Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). In the hindbrain, which is their rostral-most area of expression, *Hox* genes display sharp boundaries of expression along rhombomere boundaries and may be involved in specifying segmental fate (Wilkinson et al., 1989a; Hunt et al., 1991a). *En-2*, one of the vertebrate homeobox genes that shows sequence similarity to the *Drosophila engrailed* gene, is expressed more anteriorly, in

a narrow band of cells that spans the midbrain/hindbrain boundary (i.e. Joyner and Martin, 1987; Hemmati Brivanlou et al., 1991).

Homeobox genes of the families discussed above have been used in *Xenopus* embryos as regional markers to show that anterior chordal mesoderm can induce anterior neural tissue while posterior chordal mesoderm induces neural tissue of more posterior character (Hemmati-Brivanlou et al., 1990a; Sharpe and Gurdon, 1990; Saha and Grainger, 1992). In addition, mesoderm-inducing factors and retinoic acid have been implicated in the regionalization of the epichordal nervous system, possibly indirectly by altering the A-P character of the underlying mesoderm (reviewed in Slack and Tannahill, 1992; Papalopulu and Kintner, 1992). These results support the classical view that the regionalization of neural tissue depends on instructions from the underlying mesoderm (reviewed in Sáxen, 1989). Recent studies, however, have shown that large amounts of neural tissue are also formed under experimental conditions, such as those that occur in exogastrulae or Keller sandwiches, where the mesoderm does not involute and therefore never lies in apposition with the ectoderm (Kintner and Melton, 1987; Keller and Danilchik, 1988; Dixon and Kintner, 1989; Ruiz i Altaba, 1990, 1992). Moreover, the neural tissue formed under these conditions expresses epichordal regional markers in the correct spatial order (Doniach et al., 1992; Ruiz i Altaba, 1992). These results indicate that the induction and patterning of neural tissue may depend both on signals that pass within the plane of ectoderm as well as signals from underlying mesoderm.

In contrast to posterior regions of the nervous system, relatively little is known about the contribution of vertical and planar signals to the induction of the forebrain. On one hand, head mesoderm has been shown to be a very poor inducer of neural tissue, suggesting that forebrain induction cannot occur by vertical signals alone (Dixon and Kintner, 1989; Sive et al., 1989; Sharpe and Gurdon, 1990; Ruiz i Altaba, 1992). On the other hand, Keller sandwiches or exogastrulae rarely form eyes unless combined with head mesoderm, suggesting that formation of forebrain requires the co-operation of two signals (Dixon and Kintner, 1989; Ruiz i Altaba, 1992). Further analysis of forebrain induction has been hampered by the lack of early, forebrain-specific, molecular markers. Several genes distinct from the *Hox* genes and *en-2*, such as the *POU*-genes, (He et al., 1989), *Wnt* genes (Roelink and Nusse, 1991) and *PAX* genes (Goulding, 1992), are expressed in the forebrain but these are also expressed in more posterior regions and therefore are of limited practical value as anterior-specific markers.

Recently, a number of genes have been characterized in the mouse that show restricted expression in the forebrain (Price et al., 1991; Porteus et al., 1991; Robinson et al., 1991; Price et al., 1992; Simeone et al., 1992a, b). Here we report the isolation by PCR of a family of *Xenopus* genes (*X-dll1* to *5*) that contain a homeobox most related to that of the *Drosophila Dll* gene. cDNA clones were isolated for *X-dll2*, *3* and *4*, and their expression patterns were studied by RNase protection and in situ hybridization. This analysis has shown that, within the neural ectoderm, expression of *X-dll3* and *4* is restricted to the ventral forebrain and moreover, that *X-dll3* is a very early marker of this part of the nervous system, which is ontogenetically the most anterior part of the neural plate (Eagleson and Harris, 1989). Finally, we have shown that these anterior neural plate markers can be induced by signals that spread within the plane of the neural ectoderm. Thus, the initial steps of forebrain formation do not require signals from the underlying mesoderm, as is also the case for the epichordal neural ectoderm (Doniach et al., 1992; Ruiz i Altaba, 1990, 1992).

MATERIALS AND METHODS

Cloning by PCR

The degenerate primers that were used to amplify *Dll*-related sequences are underlined in Fig.1A and were as follows: upstream primer (corresponding to aa RKPRTIY): 5′ AG(GA) AA(GA) CC(CAT) CG(CT) AC(CAT) AT(CAT) TA 3′; downstream primer (corresponding to QVKIWFQN) : 5′ CA(GA) GT(AGCT) AA(GA) AT(TC) TGG TTC CAG AA 3′. PCR amplifications were in a 50 μl reaction containing 1 μl of crude phage lysate of a λgt10 stage 17 cDNA library (titre: $5x10^{10}$pfu/ml) as a DNA substrate, approximately 10 μg of each degenerate primer and Vent^R DNA polymerase (New England Biolabs) according to the manufacturer's instructions. Samples were denatured for 3 minutes at 94°C and amplified forty (40) times, each amplification cycle consisting of 1 minute at 94°C, 3 minutes at 37°C and 1 minute at 72°C. Samples were finally extended for 5 minutes at 72°C. The unusually low annealing temperature was based on Lai and Lemke, (1991). Amplified DNA sequences were resolved on a 5% nondenaturing acrylamide gel and fragments of the appropriate size were excised, eluted, blunt-ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, subcloned into the plasmid vector pKS$^+$ (Stratagene) and sequenced.

Isolation of *distal-less* cDNAs

PCR cDNA fragments were excised from the plasmid vector, ^{32}P-labelled and used to screen a λgt.10 cDNA library (10^{10} pfu/ml) under conditions of high stringency. The cDNA library was constructed from RNA isolated from the anterior half of stage 17 neurulae. The filters were hybridised in 50% formamide, 6× SSPE, 1× Denhardt's, 0.1% SDS and 100 mg/ml salmon sperm DNA at 42°C overnight (O/N) and then were washed in 0.1% SDS, 0.5× SSPE at 68°C for approximately 4 hours. Positive clones were plaque purified and the largest phage cDNA clones were subcloned as *Eco*RI fragments into the plasmid vector pKS$^+$(Stratagene).

Sequencing

Subcloned PCR products and cDNA clones were sequenced with the dideoxy chain termination method (T7 sequenase kit by United States Biochemical Corporation) and oligonucleotide primers.

In situ hybridisation

Embryos were fixed and processed as described in the in situ hybridization protocol of Hemmati-Brivanlou et al. (1990b). RNA probes were prepared by in vitro transcription of the linearized DNA templates shown in Fig. 1B, in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The hybridization probes for actin and *en-2* are described in Hemmati-Brivanlou et al. (1990b) and Bolce et al. (1992), respectively. The probe for *X-twi* spans the coding region (Hopwood et al., 1989) and was kindly donated by Dr D. Turner. The probe for *N-CAM* was made from a *Cla*I

linearized plasmid containing an *N-CAM* cDNA clone (N1; Krieg et al., 1989) transcribed with SP6 polymerase. In vitro transcription was also performed as in Hemmati-Brivanlou et al. (1990b). Hybridization was detected using a alkaline phosphatase-coupled, anti-digoxigenin antibody (Boehringer Mannhein) diluted to 1:1000. Alkaline phosphatase staining was developed with NBT/BCIP (Sigma; see Hemmati-Brivanlou et al., 1990b) and the reaction was allowed to proceed from 2 hours to O/N, depending on the probe. Stained embryos were fixed O/N in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), dehydrated in methanol for 1-2 hours, cleared, mounted in 2: 1 benzyl benzoate: benzyl alcohol and photographed. Some specimens were sectioned after staining, and these were fixed O/N in MEMFA, then dehydrated in methanol, permealised briefly (2×10 minutes in Xylene), followed by 2×20 minutes changes in 1:1 xylene: paraffin wax at 60°C, and embedded in Paraffin wax. Sections (10 μm) were cut, dried, dewaxed according to standard histological procedures, mounted in Permount and photographed with Nomarski optics.

RNA analysis

Xenopus embryos were fixed and dissected in ethanol: glacial acetic acid (95:5). Dissected tissue was collected into 1 ml homogenizers, and extracted for RNA as described in Bolce et al. (1992). Expression of specific RNA transcripts was detected by RNase protections, using ^{32}P-labelled antisense RNA probes, as previously described (Melton et al., 1984; Kintner and Melton, 1987). The probes used to detect *N-CAM* and *eF1-a* RNA are described elsewhere (Kintner and Melton, 1987; Dixon and Kintner, 1989).

Embryos and Keller explants

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg laying and in vitro fertilization using standard methods. Embryos were staged according to Nieuwkoop and Faber (1967). *Xenopus* embryo explants (Keller sandwiches), designed to test the effect of planar signals, were prepared as previously described in Keller and Danilchik (1988). Briefly, square pieces of tissue were dissected from the dorsal side of an early gastrula (blastopore lip of approximately 30°). Each piece included the prospective dorsal mesoderm (dorsal involuting marginal zone, DIMZ), the prospective neural ectoderm (dorsal noninvoluting marginal zone, DNIMZ) and part of the animal cap. Care was taken to exclude the lip itself and to scrape off any already involuted head mesoderm. Two such pieces were sandwiched together with their deep surfaces apposed and were cultured under glass coverslips in Sater's Modified Danilchik's medium as previously described (Doniach et al., 1992). We feel that our explants were free of underlying mesoderm because they showed two regions of convergent extension, one in the DIMZ and one in the DNIMZ, as previously described (Keller et al., 1992a,b and references therein). Keller sandwiches that contain head mesoderm fail to undergo convergent extension in their DNIMZ part (Keller et al., 1992a).

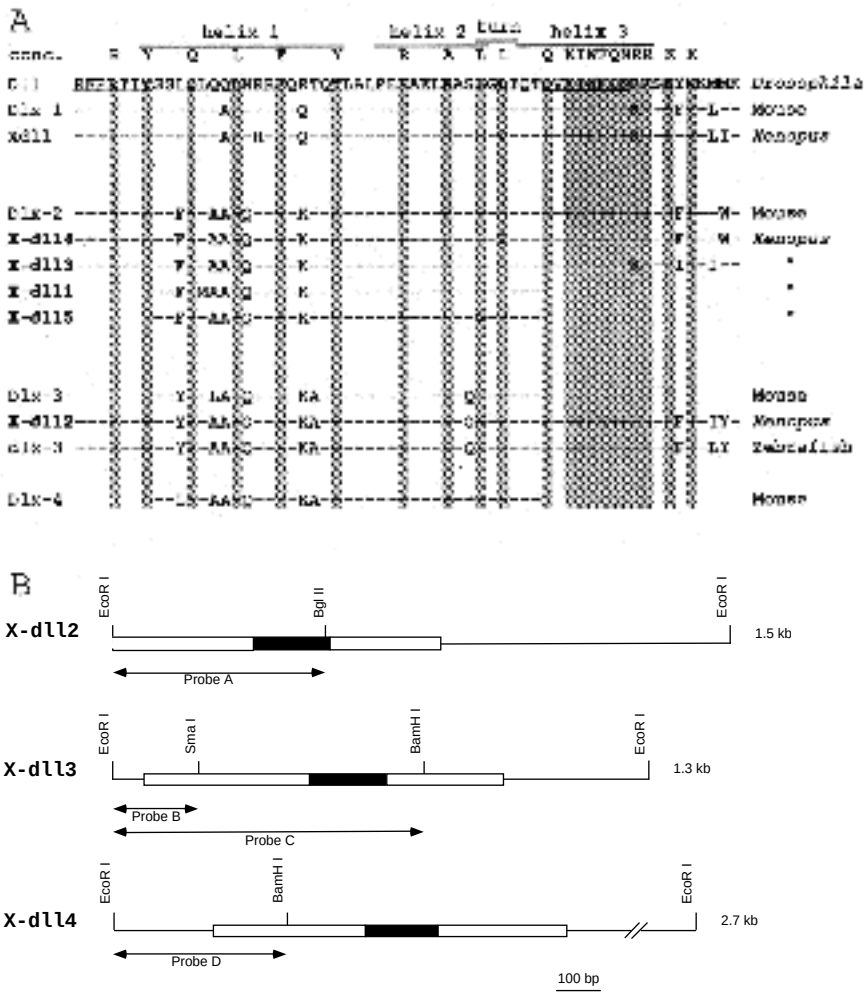

Fig. 1. (A) Diagram of the *Xenopus distal-less* homeodomain sequences reported in this paper (*X-dll1* to *5*; in bold), aligned with related genes from other organisms, as indicated on the right. The aa residues that are highly conserved in all eukaryotic homeodomains are shaded and the consensus (conc.) is shown on top. All genes are compared to the *Drosophila Dll* gene (top) and identities are indicated by dashes. The vertebrate genes have been classified according to type and position of the aa differences with the *Drosophila Dll* sequence as exemplified by the sequence of mouse *Dlx-1, 2, 3* and *4* (see text for details). Only part of the homeodomain sequence of *Xenopus X-dll1* and *X-dll5* and the mouse *Dlx-3* and *Dlx-4* is shown because only PCR fragments of these genes have been isolated. The aa sequence of the PCR primers used in this work is underlined. The sources of the sequences shown are: *X-dll1* to *5*, this work; *Dll*, Cohen et al. (1989); *Dlx-1*, Price et al. (1991); *Dlx-2*, Robinson et al. (1991); Porteus et al. (1991); *Dlx-3* and *Dlx-4*, Robinson et al. (1991); *Xdll*, Asano et al. (1992); zebrafish *dlx-3*, Ekker et al. (1992). (B) Schematic diagram of the *Xenopus distal-less* cDNA clones. Unfilled boxes represent the coding region, filled boxes the homeodomain and lines the 3′ and 5′ untranslated regions. *X-dll3* and *X-dll4* are full length clones but *X-dll2* lacks sequences from the 5′ end (box open at the left end). The subcloned fragments that were used as probes for the expression analysis are shown underneath. Probes A, C and D were used for in situ hybridization and probes A, B and D for RNase protection.

RESULTS

PCR amplification of five *Dll* related genes from a *Xenopus* cDNA library

Distal-less related sequences were amplified from a stage 17 *Xenopus* cDNA library using degenerate primers that correspond to the N-terminal (RKPRTIY) and the third helical (QVKIWFQN) portions of the homeodomain in the *Drosophila Dll* gene (Fig. 1A). PCR products with the appropriate size were cloned and sequenced, yielding five clones, *X-dll1* to *X-dll5*, which encode homeodomain sequences related to that of the *Drosophila Dll* gene. With few exceptions, the amino acid (aa) residues that are highly conserved in all higher eukaryotic homeodomains (Scott et al.,1989; shaded residues in Fig. 1A) are also conserved in the *Xenopus* proteins while aa variability is clustered around the region of helix 1 and in the 'turn' region between helix 2 and helix 3.

Fig. 1A shows the aa sequence of the homeodomains encoded by the *Xenopus* PCR fragments, aligned to the aa sequence of *Dll* related homeodomains that have been isolated from various organisms (Cohen et al., 1989; Price et al., 1991; Porteus et al., 1991; Robinson et al., 1991; Asano et al., 1992; Ekker et al., 1992). From this comparison, we have divided the vertebrate *distal-less* genes into four subfamilies exemplified by the mouse genes *Dlx-1*, *-2*, *-3* and *-4*, based on the position and type of aa changes within each homeodomain (Fig. 1A). Four *Xenopus* genes (*X-dll1*, *3*, *4* and *5*, this report) belong in the *Dlx-2* subfamily (Robinson et al., 1991; Porteus et al., 1991). These genes do not represent alleles, and the inferred aa differences are not artifacts generated by PCR, since the nucleotide difference ranges from 32 (between *X-dll3* and *X-dll4*) to 6 (between *X-dll1* and *X-dll4*) nucleotides over the homeobox region (105 nucleotides; data not shown). The fifth *Xenopus* clone that has been amplified in our PCR reaction, *X-dll2*, has been placed in the same subfamily as the mouse *Dlx-3* (Robinson et al., 1991), because they differ in only one aa over the part of the *Dlx-3* homeodomain shown in Fig. 1A.

Isolation of *X-dll4*, *2* and *3* cDNA clones

PCR-generated fragments were used as probes to screen for cDNA clones. This approach identified complete cDNA clones for *X-dll3* (1.3 kb) and *X-dll4* (2.7 kb), and a partial cDNA for *X-dll2* (1.5 kb; Fig. 1B). The predicted proteins encoded by two cDNAs (*X-dll3* and *4*) and the mouse *Dlx-2* gene are aligned in Fig. 2A. This comparison indicates that *X-dll4* is the most closely related to *Dlx-2* (64% identity) although whether or not these two genes are homologs is not yet certain, since other members of this

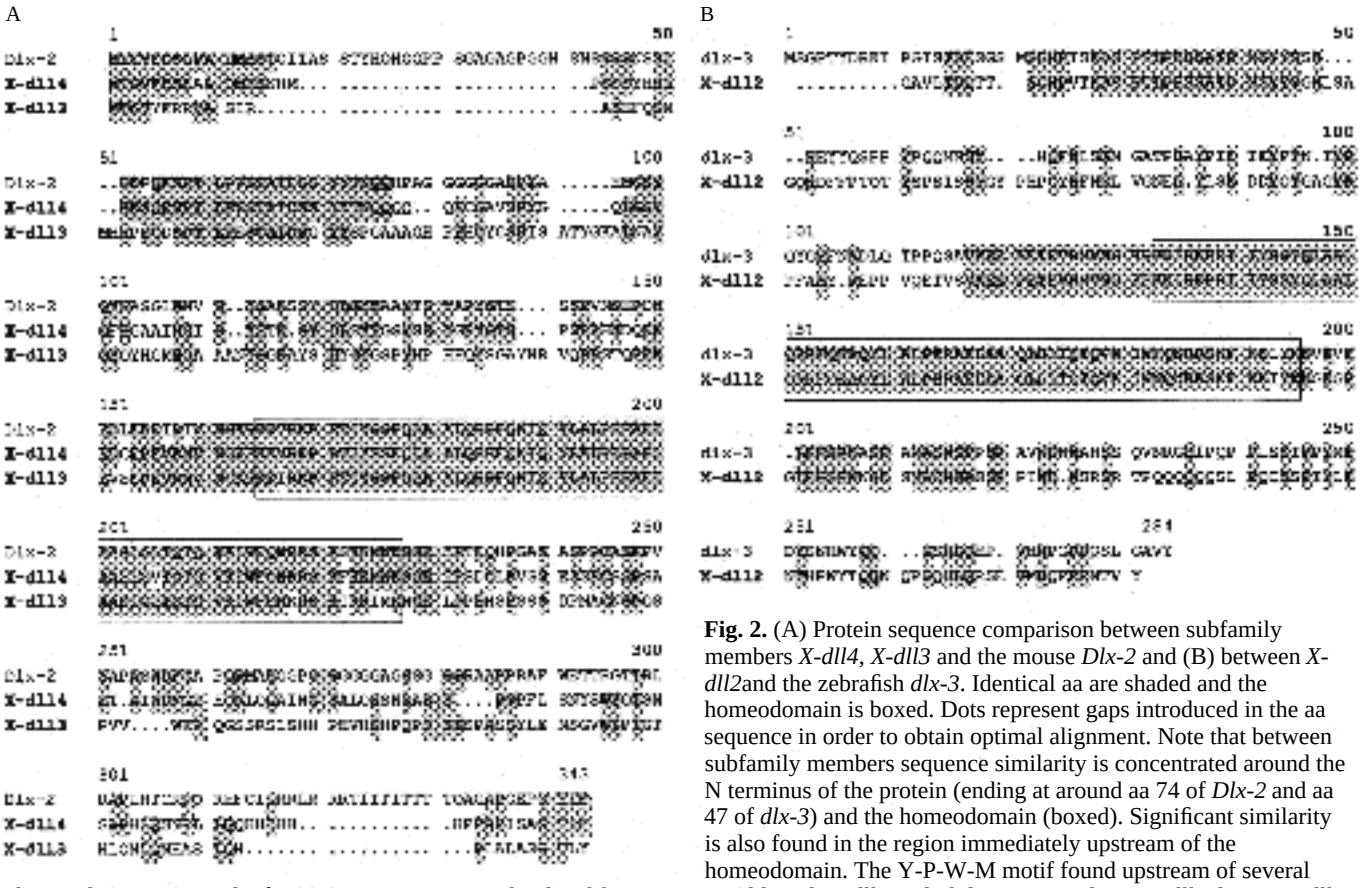

Fig. 2. (A) Protein sequence comparison between subfamily members *X-dll4*, *X-dll3* and the mouse *Dlx-2* and (B) between *X-dll2*and the zebrafish *dlx-3*. Identical aa are shaded and the homeodomain is boxed. Dots represent gaps introduced in the aa sequence in order to obtain optimal alignment. Note that between subfamily members sequence similarity is concentrated around the N terminus of the protein (ending at around aa 74 of *Dlx-2* and aa 47 of *dlx-3*) and the homeodomain (boxed). Significant similarity is also found in the region immediately upstream of the homeodomain. The Y-P-W-M motif found upstream of several homeodomains (Krumlauf, 1992) is not present in the *distal-less* proteins. Although *X-dll3* is slightly more similar to *X-dll2* than to *X-dll4* (54% and 52% at the aa level, respectively) we have placed it together with *X-dll4* in the *Dlx-2* subfamily, as the homeodomain comparison suggests (Fig. 1A), because it is more related to *X-dll4* than *X-dll2* at the nucleotide level, or at the aa level when conservative changes are taken into account. We suspect that the uncertainty regarding the placement of *X-dll3* arises from the fact that the N terminus of *X-dll2* is not included in the comparison. This issue may be clarified when the sequence of this region becomes available.

subfamily may be more related to *Dlx-2* (Fig. 1A). Similarly, *X-dll2* which was placed in the *Dlx-3* subfamily on the basis of the homeobox sequence, shows only 44% overall identity with *Dlx-2* but 63% aa identity with the zebrafish *dlx-3* gene. Thus, the sequence comparison of proteins encoded by these cDNAs supports the notion that *X-dll4* and *3* belong to one subfamily (*Dlx-2*) while *X-dll2* belongs to another (*Dlx-3*). Consistent with this interpretation is the observation that the other published *distal-less* gene from *Xenopus*, *xdll*, (Asano et al., 1992) contains a homeobox sequence that places it into the *Dlx-1* family and an overall aa sequence that is only 40% identical with the other *Xenopus distal-less* proteins, with most of the identity concentrated in the homeodomain (not shown).

The sequence similarity between *Dlx-2*, *X-dll4* and *X-dll3* shows a pattern in which regions of high sequence similarity at the N terminus and the homeodomain are separated by a more variable central region (see Fig. 2A). Also, regions of highest identity between the subfamily members *X-dll2* and zebrafish *dlx-3* are at the N terminus and the homeodomain (Fig. 2B). This pattern of sequence similarity is reminiscent of that of *Hox* subfamily genes (reviewed in Krumlauf, 1992), suggesting that the vertebrate *distal-less* genes may have also arisen by a combination of gene and locus duplication, which created related clusters of genes. In support of clustered organisation, the mouse Dlx-1 and Dlx-2 have been shown recently to be linked (McGuinness et al., 1992).

Spatial expression by RNase protection

We have analyzed the developmental expression of the *Xenopus distal-less* genes in order to determine whether any of these genes could be used as forebrain markers. Different regions of the tadpole brain were assayed for expression of the *Xenopus distal-less* genes by RNase protection assays. The sequences used as probes for each gene are indicated in Fig. 1B. Tadpole brains were dissected free of epidermis and underlying mesoderm and were subdivided into forebrain, midbrain and hindbrain (fragments 1, 2 and 3, respectively), as shown in Fig. 3. In addition, RNA was prepared from the trunk (fragment 4), tail (fragment 5) and the remainder of the tadpole, which included the eyes, branchial arches and the cement gland (fragment 6). RNA from the same number of embryos was then assayed for the presence of *X-dll2*, *3* and *4* RNA as well as for *N-CAM* RNA, a neural-specific transcript, (Kintner and Melton, 1987) or for *eF1-a* RNA, a transcript found equally in all tissues (Krieg et al., 1989). The results of this analysis (Fig. 3) indicate that *X-dll4* and *3* are expressed in the forebrain but not in the midbrain or in the hindbrain. In contrast, *X-dll2* RNA does not appear to be expressed in the forebrain or in any of the more posterior brain samples. The *X-dll3* and *4* signal in the forebrain appears low because very little tissue is present in the dissected brain regions as shown by the levels of *eF1-a* RNA. The integrity of the RNA isolated from the dissected brain regions was confirmed by probing for *N-CAM* RNA, which showed equal amounts of neural tissue present in all samples (Fig. 3).

The RNase protection analysis shown in Fig. 3 also indicated that each *distal-less* gene is also expressed at low levels in fragments 4 and 5 and at a high level in fragment

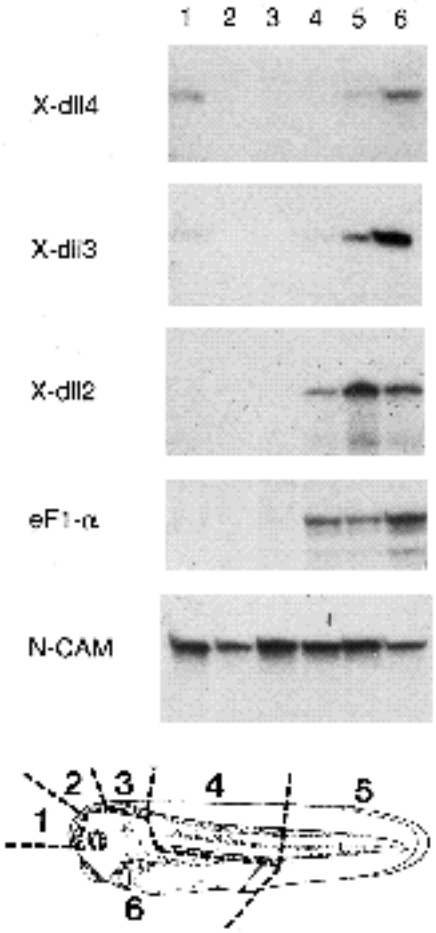

Fig. 3. RNase protection showing the spatial distribution of transcripts of *X-dll4*, *-3* and *-2*. *Xenopus* tadpoles were dissected as shown diagramatically on the right and RNA from the same number of tissue pieces were analyzed by RNase protection as shown on the left. Fragments 1, 2 and 3 (lanes 1, 2 and 3) represent the forebrain (telencephalon and diencephalon), midbrain and hindbrain, respectively. These samples were dissected free of associated structures such as eyes, epidermis and underlying mesoderm. Fragments 4 and 5 (lanes 4 and 5) represent the trunk and tail respectively including the skin, while fragment 6 (lane 6) represents the remaining of the embryo, including the eyes, branchial arches and cement gland. Since the same embryo equivalents were loaded for each lane (8 embryos/lane), the lanes corresponding to fragments 1, 2 and 3 appear underloaded when hybridized to *eF1-a*. To ensure the integrity of the RNA in these lanes, we have simultaneously analyzed the expression of the neural-specific marker *N-CAM*. The *N-CAM* signal in fragment 6 is presumably due to expression in the retina. Note that *X-dll2* is not expressed in any part of the brain (lanes 1, 2 and 3) while *X-dll4* and *X-dll3* are specifically expressed in the forebrain (lane 1). The tadpole diagram was based on Nieuwkoop and Faber (1967).

6. The very low levels of *X-dll3* in fragment 5 could be attributed by in situ hybridization to a ring of cells around the closed blastopore lip that express *X-dll3* RNA weakly and the expression of both *X-dll3* and *4* in fragment 6 could be similarly attributed to expression in the branchial arches, cement gland and the retina as discussed further below.

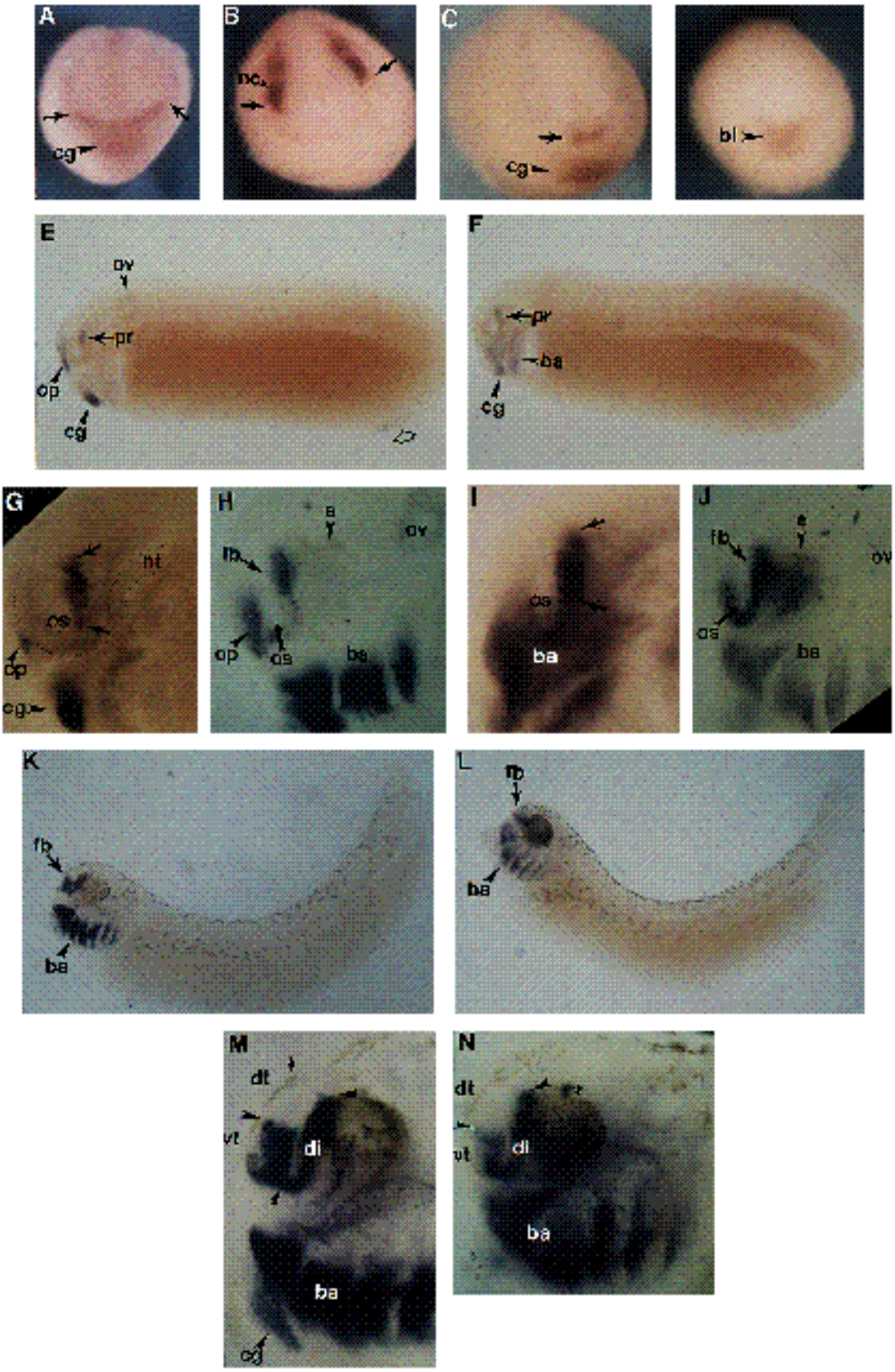
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Fig. 4

Fig. 4. Developmental expression of *X-dll4*, *3* and *2* by whole-mount in situ hybridization. In all panels apart from these on the top row, anterior is to the leftand dorsal to the top. A, C, E, G, H, K and M show the ontogenesis of *X-dll3* while F, I, J, L and N show the ontogenesis of *X-dll4*. (A) Frontal view of *X-dll3* expression at the open neural plate stage (stage 16). Staining is observed in the cement gland and the anterior transverse rim of the neural plate. (B) Frontal view of *X-twi* expression in the premigratory cranial neural crest at the same stage (stage 16). InA and B, arrows indicate the lateral limits of expression. Note that *X-twi* and *X-dll3* expression is not overlapping and if superimposed would label most of the anterior and anterolateral rim of the neural plate. (C) Frontal view of a non-cleared embryo showing the external expression of *X-dll3* a few stages later, after neural tube closure. Expression externally is detected in a band of cells, probably representing the olfactory placode (arrow) and the cement gland (cg). (D) Rear view of the same embryo as inC, showing expression of *X-dll3* around the closed blastopore lip, the prospective proctodeum (arrowhead). (E,F) Side views of cleared tailbud stage (stage 25) *Xenopus*, showing expression of *X-dll3* (E) and *X-dll4* (F). (G,I) Higher magnification of E and F. *X-dll3* is expressed in the prosencephalon, olfactory placode, otic vesicle and cement gland (E,G) and *X-dll4* is expressed in what appears to be the same region of the prosencephalon and, in addition, in the branchial arches and cement gland (F,I). The A-P limits of expression in the prosencephalon are delimited by arrows. The contour of the brain has been traced by a dashed line inG but is also visible in I. (H) Expression of *X-dll3* in the brain of a cleared stage 33 embryo. (J) expression of *X-dll4* at the same stage. Note that, in addition to the sites of expression at stage 25, *X-dll3* is now also expressed in the branchial arches (H) and *X-dll4* is also expressed in the eye (J). (K,L) Whole cleared tadpoles showing that *X-dll3* (K) and *X-dll4* (L) are expressed in the forebrain and all of the branchial arches. (M,N) Higher magnifications of *X-dll3* and *X-dll4* expression respectivelyat the same stage as in K and L. In M, small arrows point to the telencephalic-diencephalic boundary. In M and N, arrowheads point to the dorsal limit of expression in the telencephalon and diencephalon. Note that expression in the tadpole brain appears more extensive relative to expression at the tailbud stage (compare M and N with G and I; see text for details). The asterisk inN indicates a staining artifact. Abbreviations: ba, branchial arches; bl, blastopore; cg, cement gland; di, dienecephalon; dt, dorsal telencephalon; e, eye; fb, forebrain; op, olfactory placode; os, optic stalk; ov, otic vesicle; vt, ventral telencephalon.

Expression of the *distal-less* genes analyzed by in situ hybridization

The RNase protection data suggested that the neural expression of *X-dll3* and *4* is restricted to the forebrain, while *X-dll2* is not expressed in any part of the brain. In order to examine expression in greater detail, we have used whole-mount, in situ hybridization. Consistent with the results obtained by RNase protection (Fig. 3), *X-dll2* is not expressed in the brain or in any other part of the neural ectoderm (data not shown) while *X-dll3* and *X-dll4* are strongly expressed in the forebrain (Fig. 4K,L). By sectioning stage37 embryos after whole-mount in situ hybridization (Fig. 5), we have found that expression of *X-dll3* and *4* is detected on either side of the tel-diencephalic boundary, but the cells of this boundary are negative (Fig. 5O and data not shown). Expression in the diencephalon, is confined to a band of cells that appears continuous between the anterior part of the ventral thalamus and the hypothalamus (Fig. 5B,C,D; Fig. 5G,H,I; see Fig. 6 for diagrammatic summary of expression and for definition of the A-P and D-V axes). The optic stalks are negative but the chiasmatic ridge is positive (Fig. 5C,H). Other negative structures include the dorsal telencephalon, dorsal diencephalon (dorsal thalamus and epithalamus) and the bulk of the posterior part of the ventral diencephalon (posterior hypothalamus (Figs 5O, 6). However, two narrow areas of expression are detected in the posterior hypothalamus (Fig. 5E,J). In a parasagittal plane of section (Fig. 5O) these areas correspond to a punctate band of expression, (also Fig. 5N,S) that runs from the level of the ventral thalamus to the infundibular recess and terminates in the vicinity of the hypophysis (Figs 5T,O, 6), which is itself negative (Fig. 5I,M,N,S). Along the dorsoventral (D-V) axis, both genes show a sharp boundary of expression between the ventral and dorsal thalamus, the sulcus medius (Figs 5B,G, 6). Within the limits of our resolution, *X-dll3* and *4* appear to have very similar, if not identical, expression boundaries along the A-P or D-V axis. Interestingly, a difference is found along the mediolateral axis, in that *X-dll4* is expressed closer to the ventricular surface than *X-dll3* (compare *X-dll3* staining in Fig. 5B,C with *X-dll4* staining in Fig. 5G,H).

Outside the brain these two genes are expressed in complementary patterns. *X-dll3* and *X-dll4* are expressed in the branchial arches (Fig. 4H,J,K,L) but curiously *X-dll3* is restricted to the distal part of the branchial arches while *X-dll4* is expressed more uniformly and at a lower level (Fig. 5M,R). The branchial arches are populated by cranial neural crest cells, which migrate into the arches and envelope a core of muscle plate cells derived from paraxial mesoderm (Sadaghiani and Thiébaud, 1987; Noden, 1988). The pattern of staining of *X-dll3* and *4* (Fig. 5M,R) suggests that expression is restricted to the cranial neural crest, rather than the paraxial mesoderm, component of the branchial arches. *X-dll3* and *4* differ from other cranial neural-crest expressed homeobox genes (e.g. Hunt et al., 1991a,b), in that they are not expressed in premigratory neural crest (which is marked by *X-twi* expression, Fig. 4B) and are not expressed in the neural tube at the A-P level where the cranial neural crest originates, i.e. at the midbrain and hindbrain (Sadaghiani and Thiébaud, 1987; Lumsden et al., 1991 and references therein). *X-dll3* and *4* are also expressed in the sense organs of the head. *X-dll3* is highly expressed in sensory structures derived from placodes (olfactory placodes and the otic vesicle; Figs 4G,H, 5A,B,K) while *X-dll4* is expressed in the retina, a sensory stucture derived from the neural tube (Figs 4J, 5Q). It is therefore striking that the expression of these *distal-less* genes, while different, share a propensity for ectodermal derivatives of the head (forebrain, neural crest, anterior sense organs), which are exclusively vertebrate in character (Gans and Northcutt, 1983; Nothcutt and Gans, 1983). The mouse *Dlx-1* and *Dlx-2* genes are also expressed in the ventral forebrain and at a number of sites outside the neural ectoderm (Price et al., 1991, 1992; Robinson et al., 1991; Porteus et al., 1991; Dollé et al., 1992).

Ontogeny of *distal-less* expression

The results described above indicate that, within the CNS,

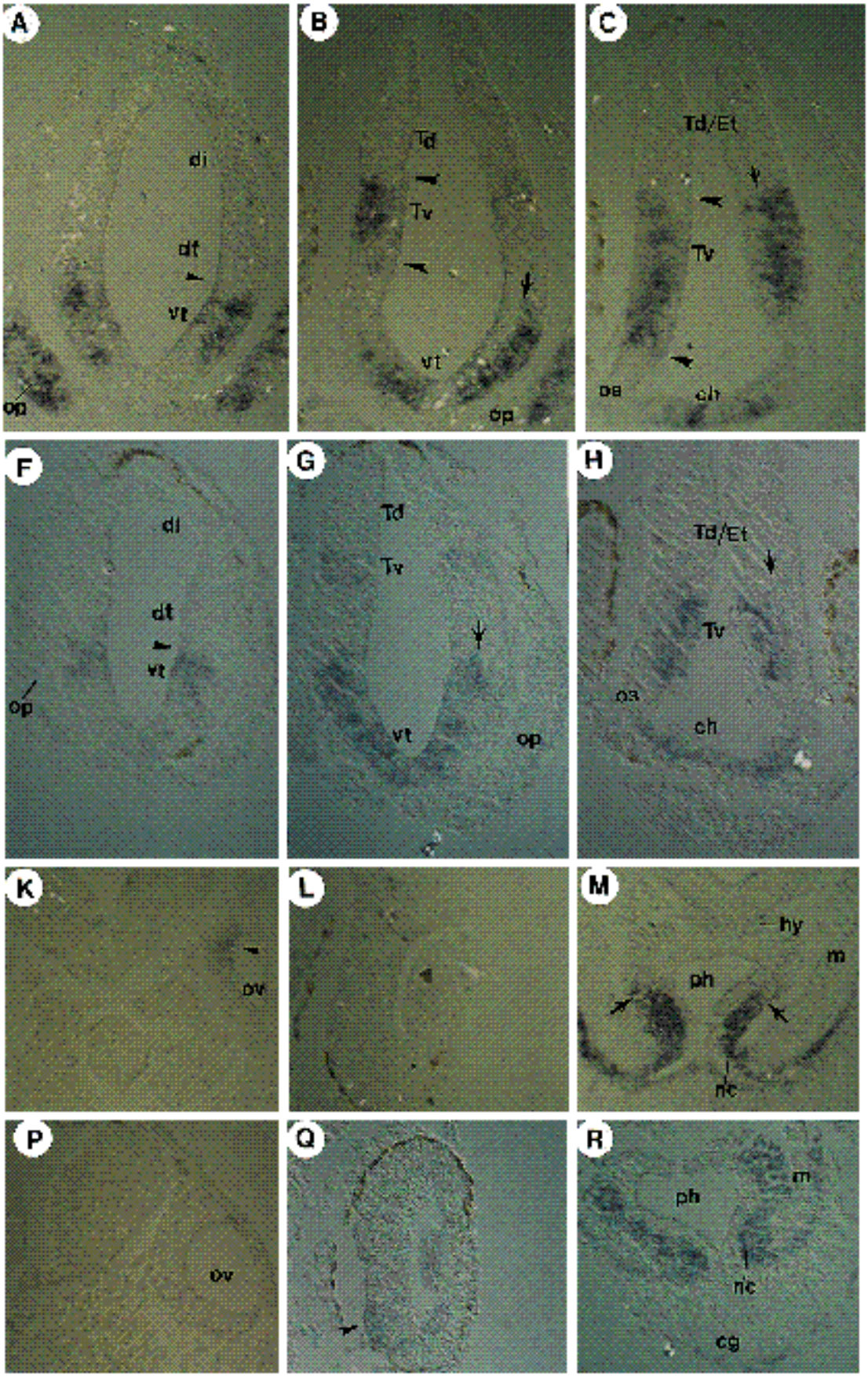
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Fig. 5

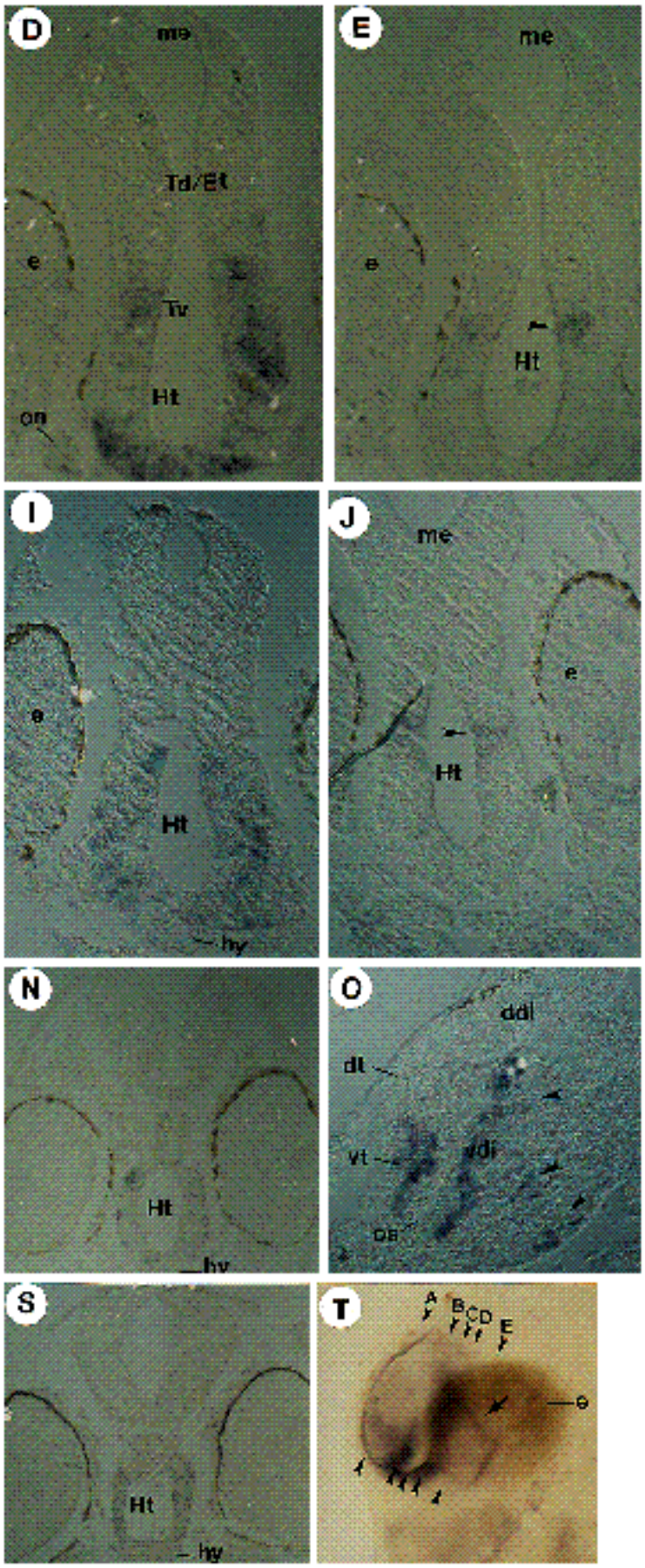

Fig. 5. Expression pattern of *X-dll3* and *4* in sections of *Xenopus* tadpoles, after whole-mount in situ hybridization. Serial sections through the brain of a stage 37 tadpole after in situ hybridization with a *X-dll3* probe (A-E) or an *X-dll4* probe (F-G). The plane of sectioning and the A-P level of the sections shown is approximately the same between A-E and F-G and is indicated by arrowheads in T, superimposed on whole-mount in situ hybridization of a stage 37 brain with *X-dll3*. For definition of the A-P and D-V axis see Fig. 6. In A and F, an arrowhead points to the dorsal limit of expression in the telencephalon. In B and C, arrowheads point to the 'segment-like' expression of *x-dll3* in the ventral thalamus. The upper arrowhead marks the sulcus medius (unlabelled) that separates the dorsal from the ventral thalamus. Note however, that expression continues ventrally in more posterior sections, into the anterior hypothalamus (B,C and D for *X-dll3*, and G, H and I for *X-dll4*). In E and J, arrowheads point to restricted punctate expression in the posterior hypothalamus. (N,S) Sections that also show the restricted expression of *X-dll3* (N) and *X-dll4* (S) in the posterior hypothalamus at a level more posterior to that shown in E and J, and at slightly older embryos (stage 39). The sections shown in E, J, N and S, pass though the punctate 'line' of expression that is indicated by an arrow in T and by arrowheads in O. (O) Parasagittal section though a stage 37 brain hybridized with *x-dll3* (the same result was obtained with *X-dll4*; data not shown) In O also note that there is non-expressing 'band' of tissue between the telencephalon and diencephalon (unlabelled), which is presumably the tel-diencephalic boundary. Although *X-dll3* and *X-dll4* appear to have the same A-P and D-V limits of expression within the neural ectoderm, they differ in that *X-dll3* is expressed more medially than *X-dll4*, as can be seen by comparing the mediolateral limit of expression (arrow) between B (*X-dll3*) and G (*X-dll4*), or between C (*X-dll3*) and H (*X-dll4)*. They also differ in their expression in the sense organs in that *X-dll3* is expressed in the olfactory placode (A and B), the otic vesicle (K) but not in the eye (L), while *X-dll4* is not expressed in the olfactory placode (F,G), and the otic vesicle (P), but is expressed in the eye (arrowhead in Q). Both genes are expressed in the branchial arch neural crest, shown in M for *X-dll3* and R for *X-dll4*, but *X-dll3* is expressed strongly only in the distal part and shows a sharp proximodistal boundary (indicated by arrows in M). Note that the neural crest envelopes a core of non-expressing mesodermally derived muscle cells. Abbreviations: ch, optic chiasma; cg, cement gland; ddi, dorsal diencephalon; di, diencephalon; dt, dorsal telencephalon; Et, epithalamus; e, eye; Ht, hypothalamus; hy, hypophysis; Tv, ventral thalamus; Td, dorsal thalamus; me, mesencephalon; m, muscle; nc, neural crest; on, optic nerve; op, olfactory placode; os, optic stalk; ov, otic vesicle; ph, pharynx; vdi, ventral diencephalon; vt, ventral telencephalon.

expression of *X-dll3* and *4* genes is localized to the forebrain. Since we were interested in using the expression of *X-dll3* and *4* to follow the development of the forebrain, the expression of these two genes was examined by in situ hybridization at the tailbud and neural plate stages of development.

The earliest stage at which we have been able to detect expression by in situ hybridization is the open neural plate stage (stage 16), for *X-dll3* (Fig. 4A). This early expression sets *X-dll3* apart from the other mouse *distal-less* genes that have been isolated thus far, in that these are expressed after closure of the neural tube (Price et al., 1991; Robinson et al., 1991). *X-dll3* is expressed in a rim of cells along the anterior-transverse ridge of the neural ectoderm (Fig. 4A), not overlapping with premigratory cranial neural crest, which stains intensely with *X-twi*, an early neural crest marker (Hopwood et al., 1989; Fig. 5B). The fate of this region is to give rise to ventral telencephalic and diencephalic structures and the olfactory placodes (Eagleson and Harris, 1989; Eagleson, personal communication), and we suggest that *X-dll3* is a very early marker for these structures. As the neural plate rolls into a tube and sinks into the embryo, cells in the anterior neural ridge are thought to segregate into the forebrain and into the olfactory placode proper (Eagleson, personal communication). *Xdll-3* staining appears to follow this segregation event upon neural tube closure in that staining is found internally in a few clusters of cells in the prosencephalon (data not shown) and externally in a mediolateral 'line' that may correspond to the olfactory placodes (Fig. 4C, and data not shown). Following neural tube closure, *x-dll4* is also detected in clusters of cells in the prosencephalon but not in presumptive olfactory placode.

In the tailbud embryo (stage 25), both genes are expressed in a restricted region of the prosencephalon (Fig. 4E,F,G,I), which lies adjacent to the anterior end of the notochord at this stage (Figs 4G, 6). Expression occurs in the region of the developing tract of post-optic commissure (TPOC; Taylor, 1991; Cornel and Holt, 1992) and it would be interesting to test whether these genes play a role in axonal guidance. Comparing the neural expression at the tailbud and tadpole stage (e.g. Fig. 4G and M; also Fig. 6), suggests that the area of *distal-less* expression expands as the forebrain enlarges. This expansion could be due to the new expression of *distal-less* genes in forebrain tissue, or to the growth and/or migration of the cells in the *distal-less*-expressing area. Outside the brain, *X-dll3* expression is found in a restricted area of the cement gland, the olfactory placodes and very weakly in a rim of cells around the proctodeum and the otocyst (Fig. 4E). *X-dll4* is also expressed in the cement gland, albeit weakly, but the placodes are negative (Fig. 4F). Instead, *X-dll4* is already expressed in the branchial arches (Fig. 4F,I). Expression of *X-dll4* in the developing eye is not detected until later, at stage 33 (Fig. 4J).

Expression in Keller sandwiches

In order to gain insight into the signals involved in forebrain induction, we have examined expression of *X-dll3* in Keller sandwiches (Fig. 7). To prepare these explants, two

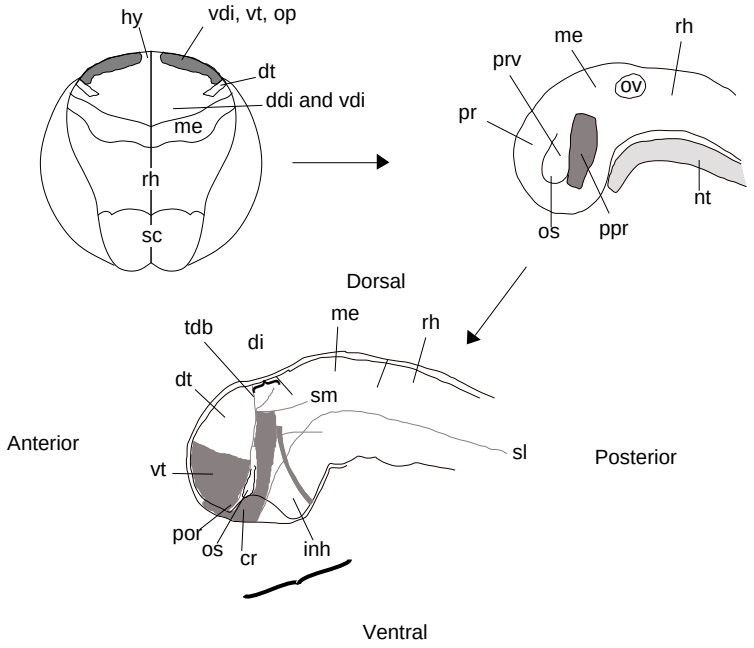

Fig. 6. Schematic summary of expression of *X-dll3* at neurula (upper left), and of *X-dll3* and *X-dll4* at tailbud (upper right) and tadpole stage (lower middle). Only expression in the neural ectoderm is shown (shaded), based on whole-mount in situ hybridization data.The A-P and D-V axis for the tadpole stages have been defined in relation to the tadpole as a whole, in contrast to other descriptions that unroll and stretch the neural tube. Therefore, the A-P and D-V nomenclature that we use reflects the position of structures in the tadpole instead of their ontogenetic relationship. The ontogenetic relationship of the tadpole's ventral and dorsal forebrain is demonstrated in the neural plate fate map (upper left) which is based on Eagleson and Harris, (1989) and Eagleson (personal communication). This map shows that part of the prospective ventral forebrain originates in a more anterior position than prospective dorsal forebrain. However, during neurulation the forebrain undergoes a rotation movement that brings rostral material in a more ventral and posterior position. Also note that the dorsal telencephalon occupies a very small area of the fate map at this stage. After closure of the neural plate, the expression pattern of *X-dll3* and *X-dll4* evolves as is shown at the tailbud (upper right) and tadpole stage (lower middle) diagram, based on tracings of Fig. 4G,I for the tailbud stage and Fig. 4M,N and Fig. 5T for the tadpole stage. The tadpole brain diagram is based on Kuhhlenbeck, 1973, vol.3, Fig. 112. The position where the sulcus limitans ends rostrally is controversial (see Kuhlenbeck, 1973), but the present interpretation is also consistent with the rostral extent of the basal plate as this is defined in Puelles et al. (1987). Abbreviations: cr, chiasmatic ridge; ddi, dorsal diencephalon; di, diencephalon; dt, dorsal telencephalon; hy, hypophysis; inh, infudibular hypothalamus; me, mesencephalon (midbrain); nt, notochord; op, olfactory placode; os, optic stalk; ov, otic vesicle; por, preoptic recess; ppr, posterior prosencephalon; pr, prosencephalon; prv, prosencephalic ventricle; rh, rhombencephalon (hindbrain); sc, spinal cord; sl, sulcus limitans; sm, sulcus medius; tdb, tel-diencephalic boundary; vdi, ventral diencephalon; vt, ventral telencephalon.

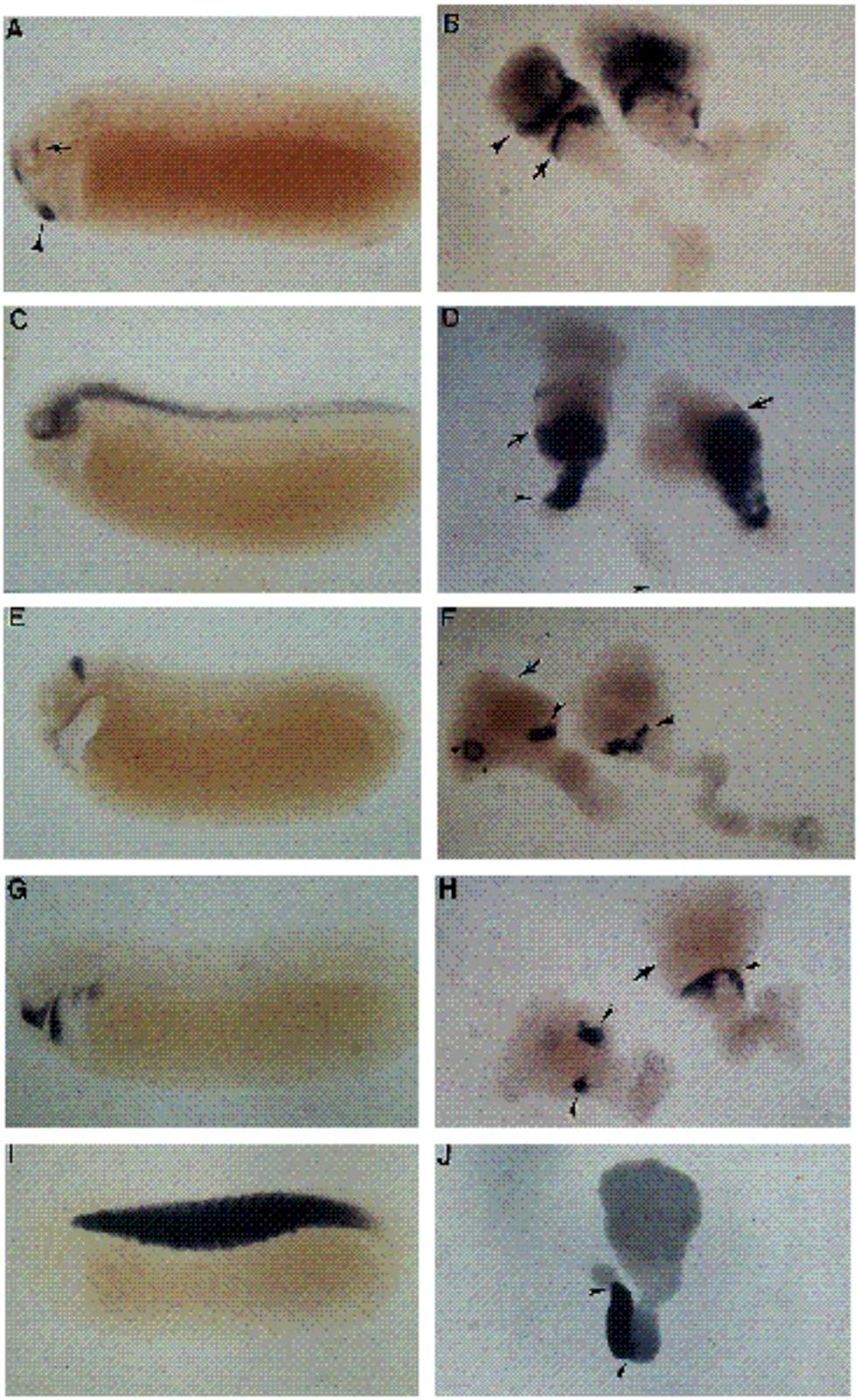

Fig. 7. Induction of gene expression by planar signals. Expression in tailbud stage embryos is shown on the left column (anterior to the left) and expression in age-matched Keller sandwiches is shown on the right column (anterior to the top). (A,B) *X-dll3*; (C,D) *N-CAM*; (E,F) *En-2*; (G,H) *X-twi*; (I,J) *Actin*. In A and B, arrows point to expression in the forebrain and arrowheads to expression in the cement gland. A higher magnification of A (Fig. 4G) shows that the level of *X-dll3* expression in the prosencephalon is comparable to the level in cement gland and olfactory placode. Note that expression of *X-dll3* in a Keller sandwich occurs around the edge of the non-elongated part of the neural ectoderm. In D and J, the limits of the mesoderm are denoted by arrowheads, illustrating that in a Keller sandwich the mesoderm forms away from the neural ectoderm. In D, the anterior limit of *N-CAM* hybridization is marked by an arrow and roughly the same level has been marked by arrows in B, F and H. In H, note that the *X-twi* hybridization is localized close to the base of the non-elongated part of the neural ectoderm (arrowheads), at about the same A-P level with *en-2* (arrowheads in F). Both markers are clearly more posterior than *X-dll3* (compare B with F and H). The staining denoted by an asterisk in F is an artifact.

square sheets of tissue are dissected from the dorsal side of the early gastrula and are combined with their deep surfaces apposed (see Materials and Methods). Such explants were cultured until stage 25, at which point we analyzed expression of various markers by whole-mount in situ hybridization. In these sandwich explants, the dorsal mesoderm moves away rather than underneath the ectoderm, thus allowing the study of signals propagating within the plane of the ectoderm (Fig. 7D,J; Keller et al., 1992a,b).

In these explants, *X-dll3* is expressed in two, nearly par-

allel, stripes (Fig. 7B). One stripe (arrowhead in Fig. 7B) appears to correspond to the cement gland based on the cellular morphology, the secretion of mucus and the fact that it occurs well outside the neural ectoderm (as shown by double label *N-CAM* staining; data not shown). The neural ectoderm of the Keller sandwiches, consists of a elongated region (posterior) and a rounded, distal region (anterior) (*N-CAM* staining in Fig. 7D). The second stripe of *X-dll3* staining occurs around the rim of the rounded distal region of the neural ectoderm (arrow in Fig. 7B) and is therefore more anterior than *en-2*, which is expressed closer to the elongated neural region (Fig. 7F). One might argue that this stripe of *X-dll3* expression can be attributed to expression in the cranial neural crest. This is unlikely, first because *X-dll3* is not expressed in cranial neural crest at the stage of analysis (control stage 25; Fig. 7A), and second because a neural crest marker, *X-twi*, (Fig. 7G; Hopwood et al., 1989) is not expressed in this region. In contrast, as shown in Fig. 7H and F, *X-twi* is expressed more posteriorly, around the same level as *en-2*, which is consistent with the A-P level of origin of the cranial neural crest (Lumsden et al., 1991, and references therein). One might also argue that this *X-dll3* staining solely represents expression in the sense organ anlage such as the olfactory placode or the otic vesicle. However, the same pattern of hybridization in a Keller sandwich was obtained with *X-dll4*, which is not expressed in these sensory anlage (data not shown). Because the same result was obtained with both markers, we conclude that the rim-staining in a Keller sandwich includes part of the forebrain anlage. Thus, these results suggest that in addition to epichordal neural plate markers, planar interactions are responsible for inducing markers of the rostral-most part of the neural plate.

DISCUSSION

Xenopus distal-less genes are expressed in forebrain, cranial neural crest and sensory organs

In contrast to *Drosophila* where only one *Dll* gene has been reported (Cohen et al., 1989), the analysis of *distal-less* genes in the mouse, *Xenopus* and zebrafish (Price et al., 1991; Porteus et al., 1991; Robinson et al., 1991; Asano et al., 1992; Ekker et al., 1992), suggests that vertebrates contain a number of *distal-less* genes, which we have subdivided into 4 subfamilies. Sequence similarity places four of our five PCR clones into one subfamily (the *Dlx-2* subfamily) and the fifth PCR clone into another (the *Dlx-3* subfamily).

RNA corresponding to *X-dll2*, a member of the *Dlx-3* subfamily, did not appear to be localized in neural tissue either when assayed by RNase protection or by in situ hybridization. *X-dll2* and the recently described related zebrafish gene, *dlx-3* (Ekker et al., 1992), together with the mouse *Dlx-3*, define a distinct subfamily which may not be expressed in the nervous system. *X-dll3* and *4*, members of the *Dlx-2* subfamily, are expressed in the tadpole nervous tissue and their expression is restricted to the forebrain as measured by both RNase protection and in situ hybridization. Expression in the forebrain is confined to the ventral telencephalon and anterior ventral diencephalon (see Fig. 6 for diagramatic representation of expression and for definition of A-P and D-V axis). Although ventral in position, areas of the tadpole brain that express *X-dll3* and *X-dll4* are likely to be derivatives of the alar and not the basal plate, as has been previously suggested for the mouse Dlx-2 gene (Robinson et al., 1991). In most of the nerve cord, ventral and dorsal neural tissue corresponds to basal and alar plate, respectively. This distinction is less clear in the forebrain because of the bending of the neural axis during neurulation and because of the uncertainty in the course of the sulcus limitans, which separates basal and alar plate in the posterior nervous system. Recent studies suggest, however, that the rostral limit of the basal plate stops in the middle of the chiasmatic ridge (Puelles et al., 1987, and see Fig. 6), in agreement with the course of the sulcus limitans suggested by others (see discussions in Kappers et al., 1936 vol. 3, pp 1240-1242; Bergquist and Källén, 1954; Kuhlenbeck, 1973; vol. 3, pp 289-304). Therefore, in this view, most of the *X-dll3* and *4* expression lies in alar forebrain, while only the limited area of expression that occurs in the posterior (infundibular) hypothalamus would fall into the basal plate.

X-dll3 expression marks the anterior extent of the neural plate, which gives rise to the ventral forebrain

Our results show that expression of *X-dll3* can be detected in embryos by in situ hybridization as early as the open neural plate stage of development where staining is localized to a thin transverse crescent along the rim of the anterior neural plate (Figs 4A, 6). We propose that the region of the anterior transverse ridge stained with *X-dll3* is likely to be the anlage for the ventral telencephalic and diencephalic brain structures and for the olfactory placodes that express *X-dll3* at tadpole stages. First, a recent fate map of the *Xenopus* neural plate shows that the anterior neural ridge gives rise to ventral telencephalic and diencephalic brain structures that express *X-dll3* (Eagleson and Harris, 1989). Second, in a number of vertebrate species, the anterior neural ridge includes the prospective olfactory placodes, derived from the nervous layer of the ectoderm which is topographically located at the anterior boundary of, and is continuous with, the neural plate (Klein and Graziadei, 1983; Knouff, 1935; Jacobson, 1959; Couly and LeDouarin, 1988; van Oostrom and Verwoerd, 1972). In addition, recent tracing studies have shown that the *Xenopus* neural ridge give rises to cells in both the olfactory placode and the ventral forebrain (Eagleson, pers. comm). Later on, the olfactory placode continues to contribute cells to the forebrain (reviewed in Schwanzel-Fukuda and Pfaff, 1990). Finally, the telencephalon fails to develop following an early removal of the olfactory placode anlage (Graziadei and Monti-Graziadei, 1992). Thus, the early expression of *X-dll3* in the anterior transverse ridge of the open neural plate supports the notion that there is a close topographical and functional association between cells of the olfactory placode and the forebrain. During neurulation, cells that are located in the anterior transverse ridge segregate into the brain while others give rise to the olfactory placode proper (Eagleson, personal communication), and both structures express *X-dll3* after neurulation is completed. In

contrast, *X-dll4*, which is not expressed in the olfactory placode, is not expressed in the forebrain until after the segregation of forebrain from olfactory placode cells, that is after closure of the neural tube. Finally, the punctate expression of *X-dll3* and *X-dll4* in parts of the posterior hypothalamus may be due to the extensive migrations that occur in the hypothalamic-hypophyseal area (Eagleson and Harris, 1989).

In conclusion, we suggest that *X-dll3* expression marks the rostral-most part of the neural ectoderm at the open neural plate stage (Fig. 6). In addition, the expression of *Xenopus distal-less* in the forebrain may be ontogenetically related to expression in the neural crest in that the anlage of both are located along the rim of the neural plate, at an anterior and anterolateral position, respectively (Sadaghiani and Thiébaud, 1987; Couly and LeDouarin, 1988; also seen by juxtaposing the *X-dll3* and *X-twi* staining in Fig. 4A,B).

Expression of *X-dll* genes and segmentation in the forebrain

Neuromeric segmentation has been shown to play an important role in the development of the hindbrain, where cell lineage (Fraser et al., 1990) and the early expression of molecular markers (e.g. *Hox* genes, Wilkinson et al., 1989a; *Krox-20*, Wilkinson et al., 1989b) appear to respect segment boundaries. Since several authors have proposed that neuromeric segmentation might also play a role in the development of the forebrain (see Puelles et al., 1987, and refs. therein), it is of interest to know whether the restricted expression of the *Xenopus distal-less* genes in the forebrain corresponds to the morphological boundaries that occur at neuromeric segments, as suggested for the mouse genes (Price et al., 1991, 1992; Robinson et al., 1991).

Although the exact number and location of neuromeres in the anterior end of the nervous system is not universally agreed upon (see discussion in Puelles et al., 1987), we have roughly diagrammed these boundaries onto the tadpole brain as shown in Fig. 6, and placed the distribution of *X-dll3* and *4* RNA in the forebrain onto this diagram, using data from whole-mount in situ hybridization and coronal sections. This comparison shows that the expression of these two distal-less genes does observe some segment boundaries. First, the expression of *X-dll 3* and *4* respects a boundary that occurs between the dorsal and ventral telencephalon (Fig. 6), which supports Berquist and Källén's model of subdividing the brain (see summary of models of segmentation in Puelles et al. (1987)). A second boundary that appears to be respected in the diencephalon is the sulcus medius, which separates the dorsal from the ventral thalamus (Herrick, 1910; reviewed in Kuhlenbeck, 1973) and is apparently topographically close to the zona limitans intrathalamica of Puelles et al. (1987). Expression of both genes is continuous ventrally until the region of the optic chiasm, and therefore spans at least one segment. The correlation with morphological segmentation is complicated by the fact that the expression of *X-dll3* and *4* is restricted to a narrow portion of the diencephalic segments along the A-P axis of the tadpole (see Fig. 6), giving the impression that expression runs perpendicular to the transverse boundaries in the diencephalon. In this respect, expression of *X-dll3*and *X-dll4* may support the segmentation model of Berquist and Källén (1954) that subdivides the brain into longitudinal columns and transverse bands.

In sum, the *X-dll3* and *4* expression in the forebrain does respect some segment boundaries but is not specific for any one segment and it does not follow neuromeric segmentation in a simple way. As an alternative model, we propose that complex pattern of *distal-less* expression at the tadpole brain is derived from a rather simple pattern of expression at the neural plate stage (see above). Thus, we suggest another level of regionalisation where the forebrain is subdivided into different regions at the neural plate stage, as marked by the expression of *X-dll3*. This model can be tested by tracing the neuromeric boundaries back to the neurula stage in order to examine their correlation with early *X-dll3* expression.

Planar signals induce anterior neural plate

At the tailbud stage, the anterior notochord is in close vicinity with the site of *X-dll3* and *4* expression in the brain (see Figs 4G, 6), offering the possibility that the expression of *X-dll3* and *X-dll4* in the forebrain could be established by 'vertical' signals emanating from the underlying chordal mesoderm. To address the role of the notochord in the induction of the forebrain anlage, we have analyzed *distal-less* expression in Keller sandwiches, where inducing signals pass within the plane of ectoderm rather than vertically between ectoderm and underlying mesoderm. We have shown that *X-dll3* is expressed in a Keller sandwich around the anterior rim of the neural ectoderm, which would give rise to the ventral forebrain and the olfactory placodes. This staining can be identified unambiguitously as including the anlage of the ventral forebrain based on comparison with other markers (see results). These results demonstrate that midline/notochord signals are not necessary, and that planar signals are sufficient to induce anterior neural plate markers such as *X-dll3*. These results are consistent with, and extend, previous studies that have shown that large amounts of neural tissue can form via planar signals (Kintner and Melton, 1987; Keller and Danilchik, 1988; Dixon and Kintner, 1989) and that this neural tissue is patterned along the A-P axis as measured by the correct spatial expression of genes that mark the different A-P levels within the epichordal nervous system (Doniach, 1992; Ruiz i Altaba, 1992). Thus, the induction of the anterior neural plate is brought in line with that of more posterior, epichordal, parts of the nervous system, in that they both rely on signals propagating within the plane of the ectoderm rather than signals from underlying notochord/head mesoderm.

Dixon and Kintner (1989) and Ruiz i Altaba (1992) showed using morphological markers that planar signals need to act synergistically with vertical signals from the underlying head mesoderm in order to obtain structures characteristic of the head of a normal embryo (e.g. eyes). Our findings have shown that, although head structures may not differentiate in a Keller sandwich, planar signals are sufficient to induce anterior neural plate-expressed genes such as *X-dll3*, in the correct A-P spatial order in relation to epichordal gene markers (Fig. 7 and Doniach et al., 1992). Vertical signals from the prechordal mesoderm or from the anterior notochord are presumably needed in order

for the anterior neural ectoderm to differentiate further and to give rise to a morphologically recognizable head. Keller sandwiches also lack pigment cells (Keller, 1992a), consistent with the notion that an interaction with underlying dorsal lateral mesoderm is necessary for neural crest formation (Mitani and Okamoto, 1991). However, by using a neural crest-specific marker, we have shown that cranial neural crest does form in these explants, at an A-P level that corresponds to its origin in vivo (Sadaghiani and Thiébaud, 1987). Perhaps, as in the case of forebrain, vertical signals are essential for further differentiation of neural crest derivatives.

Vertical signals are also needed for morphogenetic events such as the rolling of neural plate into a tube, a process that is not thought to take place in a Keller sandwich (Keller and Danilchik, 1988; Keller et al., 1992a). Indeed, we have noted that, in a Keller sandwich at the control tailbud stage, *X-dll3* is still expressed in a transverse, slightly curved, stripe, resembling the normal expression of the gene at the open neural plate stage (compare Figs 4A and 7B). Failure of the neural tube to close may account for the observation that in a Keller sandwich the separation of *X-dll3* staining in the olfactory placodes from ventral forebrain does not take place.

In conclusion, the results from Keller sandwiches indicate that forebrain induction and patterning may occur by planar signals, although the nature and source of these signals remain unknown. It is intriguing that, at the neural plate stage, the shape of the forebrain and cranial neural crest anlage, which both stain with *X-dll3* (albeit at different developmental stages), is semi-circular, perhaps suggesting that planar signalling at the anterior neural plate is radial in orientation.

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