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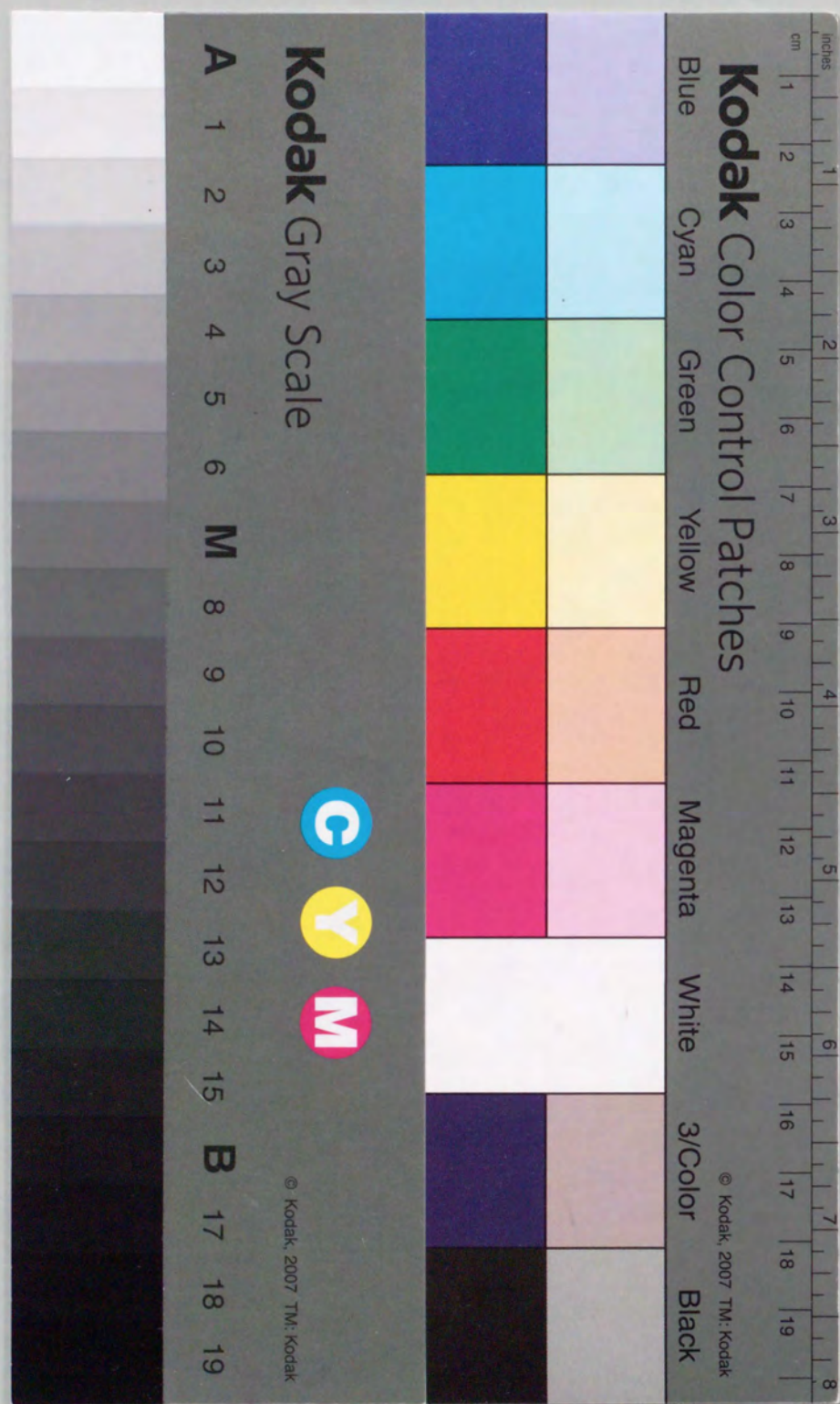
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Regionalized expression of the *Dbx* family homeobox genes  
in the embryonic CNS of the mouse

HIROKI SHOJI

1996





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by  
Hiroki Shoji

THESIS



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## Summary

Here I report the identification of a novel homeobox gene family *Dbx* in mouse, which consists of *Dbx* and *Dbx2*. The two genes share similar structural organization and are encoded by different chromosomes. The predicted *Dbx* and *Dbx2* proteins share 85% identity in their homeodomain amino acid sequences, but otherwise showed no significant similarity. Characterization of the expression of these two genes in the embryos suggested their role in the development of the CNS. In the forebrain, *Dbx* is expressed in various regions, while *Dbx2* showed a more restricted pattern of expression. In the midbrain, the expression domains of *Dbx* and *Dbx2* overlap along the dorso-lateral wall of the ventricle. In the hindbrain and spinal cord, both genes are expressed in the boundary separating the basal and alar plates, which seems to correspond to the sulcus limitans. Expression of the *Dbx/Dbx2* genes is restricted to the ventricular region of the embryonic CNS except for that of *Dbx* in the septum of the telencephalon. Together these observations indicate possible participation of the members of the *Dbx* family in regionalization of the CNS. While the expression of *Dbx* was restricted to the CNS, *Dbx2* was also expressed in some of the mesenchymal cells, such as limb buds and tooth germs.



## Introduction

Our understanding of the molecular mechanisms underlying embryonic morphogenesis has been accelerated by genetic studies achieved in *Drosophila melanogaster*. Number of regulatory genes has been identified which have been integrated into a cascade of genetic networks (for review, Akam, 1987). Homeoboxes were discovered as highly conserved 180 bp sequence motifs shared by a number of genes involved in morphogenesis (McGinnis, et al., 1984; Scott and Weiner, 1984). Homeoboxes are translated into homeodomains which serve as DNA binding domains in transcriptional regulation (for reviews, Levine and Hoey, 1988; Scott et al., 1989; Hayashi and Scott, 1990; Gehring et al., 1994).

Homeoboxes are highly conserved among various animal species not only in terms of the basic feature of coding amino acid sequences but also in the genomic composition of the homeobox-containing genes. In several animal species, a family of homeobox-containing genes have been identified, and classified into subfamilies based upon the amino acid sequences of their homeodomains. In mouse, nearly a hundred homeobox genes have been identified (Kappen et al., 1993). Murine *Hox* genes are organized into four analogous gene clusters on different chromosomes, and are differentially expressed with individually unique rostro-caudal specificity in various embryonic anlagen depending on the position within the clusters analogous to the *Drosophila Hom-C* (for reviews, Kessel and Gruss, 1990; Kenyon, 1994; Krumlauf, 1994). Other homeobox genes, such as *Pax* and *Evx1* are specifically expressed along the dorso-ventral axis of the CNS (Gruss and Walther, 1992; Bastian et al., 1990).



Involvement of the homeobox genes in regional specification of the embryonic anlagen, as suggested by analysis of their embryonic expression patterns, have been demonstrated by the phenotypes of the mutant animals lacking function of one of these genes (for reviews, Gruss and Walther, 1992; McGinnis and Krumlauf, 1992; Krumlauf, 1994).

The majority of vertebrate homeobox genes have been identified by their similarity to *Drosophila* homeobox genes. In order to identify homeobox genes including those which are highly divergent from *Drosophila* genes, I screened a mouse genomic library using a set of degenerated oligonucleotides corresponding to conserved motif in the homeodomain. This enabled me to isolate a new family of homeobox genes named *Dbx* (developing brain homeobox genes) which consists of *Dbx* (Lu et al. 1992) and *Dbx2*. These two genes are similar in their homeobox sequences and in gene organization, and are both expressed in the embryonic CNS with regional specificities.



## Materials and Methods

### Genomic and cDNA libraries

BALB/c mouse liver DNA was partially digested with *Sau3AI* and fractionated by sucrose density gradient centrifugation. Fragments with the size of 15-20 kb were ligated to  $\lambda$ EMBL3 vector arms (Clontech) and packaged *in vitro* (Stratagene). cDNAs for polyA<sup>+</sup> RNAs from 12.5 day ICR mouse embryos were methylated by *EcoRI* methylase, ligated to *EcoRI* linkers and digested with *EcoRI*. cDNAs larger than 500 bp were selected by Sepharose-CL4B column chromatography, and cloned into a  $\lambda$ gt10 vector. Screening of the genomic library using the synthetic oligonucleotide probe was done in hybridization buffer of 6 $\times$  SSC, 0.1% SDS, 0.2% Denhardt's solution, 100  $\mu$ g/ml denatured salmon sperm DNA, at 55 °C overnight, followed by 3 washes with 2 $\times$  SSC, 0.1% SDS for 15 min at 55 °C. Screening for isolation of *Dbx2* genomic clone (gS4) was done using as probe the 0.56 kb fragment of *Dbx* cDNA clone (c164) (c in Fig. 1) in the same buffer at 65 °C, followed by two washes with 1 $\times$  SSC, 0.1% SDS for 30 min at 65 °C. Screening of the cDNA library using a 0.75 kb *EcoRI*-*Bam*HI fragment of g164 or a 0.2 kb *EcoRI* fragment of gS4 as probes (a and f in Fig. 1) was done in the same hybridization condition as described above for gS4, followed by two washes with 0.1 $\times$  SSC, 0.1% SDS for 30 min at 65 °C.

### DNA sequencing

DNA was sequenced by the dideoxy method (Sanger et al., 1977) using Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical).

### Chromosomal mapping



Intersubspecific backcross progeny were generated by mating (C57BL/6 × MSM) F<sub>1</sub> females to MSM males (kindly provided by Drs. K. Moriwaki and N. Miyashita). DNA isolation, polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis were performed as described (Dietrich et al, 1994). The primer sequences used for D15Mit15 and D15Mit40 were the same as described (Dietrich et al., 1994).

### *In situ* hybridization

*In situ* hybridization with sections was done essentially as described previously (Wakamatsu and Kondoh, 1990). RNA probes were synthesized *in vitro* by transcription of a linear DNA template of 3' untranslated regions of c164 and gS4 (d and e in Fig. 1) using 200 µCi [<sup>35</sup>S]-UTP and T7 RNA polymerase (Toyobo). Paraffin sections of 6.5-15.5 day embryos of ICR mice were prepared on glass slides. The slides were treated with 0.2 N HCl and proteinase K, and then acetylated. Hybridization was done for 20 hours at 50 °C in 50% formamide, 0.2% Denhardt's solution, 2× SSC, 10 mM EDTA, 10% Dextranulphate, 500 µg/ml denatured salmon sperm DNA, 500 µg/ml yeast tRNA, 10 mM DTT, and with <sup>35</sup>S-labelled RNA probes. After hybridization, the slides were treated with 20 µg/ml RNase A and 1 unit/ml RNase T1 at 37 °C for 30 minutes, and washed in 0.1x SSC, 20% formamide, 10 mM DTT at 50 °C overnight. Autoradiography was done by exposing the slides to Kodak NTB-2 emulsion for 1-2 weeks at 4 °C and by developing with Konikadol X (Konika).

Whole-mount *in situ* hybridization of 9.5 day embryos was performed as described by Sasaki and Hogan (1993) for *Dbx*. However, hybridization signal with *Dbx2* was too faint to detect, so a procedure described by Kobayashi et al. (1994) was employed to improve signal







## Results

### Genomic and cDNA clones of *Dbx* gene family

A  $\lambda$ EMBL3 mouse genomic library was screened by hybridization with synthetic oligonucleotides representing the most conserved region of the homeoboxes [(A/G)TCAAG(A/G)TCTGGTT(C/T)CA(A/G)AA(C/T)(C/A)GG(C/A)G(C/G)ATG AAGGA] under mild stringency. From  $8 \times 10^5$  plaques, three positive clones, g161, g164 and g169, were obtained. Restriction analysis and nucleotide sequence determination indicated that g161 and g169 corresponded to *HoxB1* (Frohman et al., 1990) and *HoxD3* (Lonai et al., 1987), respectively, while g164 represented a novel gene (Figs. 1A and 2A).

To isolate the corresponding cDNA, I screened a  $\lambda$ gt10 12.5 day mouse embryonic cDNA library using a fragment of g164 as probe. The cDNA clone obtained, c164, was 1795 bp long, contained a poly A tail which was accompanied by a polyadenylation signal (AATAAA) (Figs. 1A and 2A). The longest open reading frame (ORF) found in the c164 sequence began at the 5' end and terminated at nucleotide position 830. Comparison of the c164 and g164 sequences indicated that a putative translational initiation site conforming to Kozak's consensus (Kozak, 1986) existed 171 bp upstream of the 5' end of c164 (Fig. 2A). Translational initiation at this site is consistent with the size of the transcript estimated from Northern analysis (2 kb, data not shown).

To determine whether homeobox sequences related to the g164 gene exist in mouse and chicken genomes, I used a fragment of c164 which included the homeobox (b in Fig. 1) in Southern hybridization analysis. In addition to the predicted g164 fragments, several different fragments of mouse DNA hybridized to the probe (data not shown).



Multiple hybridizing bands were also obtained with chicken DNA. These results suggested that g164 and related genes form a gene family.

Further screening of the  $\lambda$ EMBL3 mouse genomic library with a c164 fragment as probe, resulted in two positive clones. One of them corresponded to *Hlx* (Allen et al., 1991), while the other (clone gS4) represented a novel gene (Figs. 1B and 2B).

I screened the 12.5 day mouse embryonic cDNA library using a restriction fragment of gS4 as probe. One clone, cS4 consisted of a 1035 bp insert with a 703 bp ORF which was missing an ATG at the 5' end but contained a poly A tail at the 3' end (Figs. 1B and 2B).

The nucleotide sequences and exon-intron boundaries of the genes represented by g164 and gS4 were reconstructed with the aid of their cDNA sequences, and are shown in Fig. 2. After these sequences were determined, Lu et al. (1992 and 1994) reported a new gene *Dbx* (developing brain homeobox gene) which was identical to the gene for g164/c164. There were several sequence disagreements in the 5' and 3' noncoding regions between the cDNA sequence reported by Lu et al. (1994) and g164 (Fig. 2A, nucleotides 799, 3027, 3096-8, 3409, 3415, 3517, 3565-6), which may reflect polymorphism or sequence error. gS4 which was entirely new in the mouse, I named *Dbx2*.

The amino acid sequence conservation between the homeodomains of *Dbx* and *Dbx2* is 85% (Fig. 3). In addition, a significant similarity can be found in the 15 amino acids located C-terminal to the homeodomains. Comparison of the genomic and cDNA clones of *Dbx* indicated that the gene consists of at least 4 exons. The structure of *Dbx2* has not been completely resolved but the position of the intron/exon junctions in the homeobox was identical to that of *Dbx*



(Figs. 1 and 2). The structural similarity and expression specificity to be described below indicated that *Dbx* and *Dbx2* compose a gene family.

Comparison of the amino acid sequences of *Dbx* and *Dbx2* homeodomains with those of other proteins revealed that the zebrafish *hlx-1* (Fjose et al., 1994) and the chicken *CHoxE* (Rangini et al., 1991) appeared to be homologues of *Dbx* and *Dbx2*, respectively. *hlx-1* shared a 97% similarity with *Dbx* and *CHoxE* a 93% similarity with *Dbx2* in the homeodomain (Fig. 3). I have isolated a chicken clone encoding a homeobox sequence that is more homologous to *Dbx* than *Dbx2* or *CHoxE* (data not shown), suggesting that the *Dbx* gene family is conserved evolutionarily in both mouse and chicken genomes.

#### Chromosomal location of *Dbx2*

The mouse chromosomal location of *Dbx2* was determined by intersubspecific backcross analysis. The 3' intron of the gene contained a (CA)<sub>n</sub> repeat (Fig. 2B) which in many cases gives rise to genetic variation. I synthesized two oligonucleotide primers, 5' CACCCTGCACATAAACATGTG 3', 5' CTAGTAGAGCACCTATGTGTG 3', that spanned a 164 bp region containing the CA repeat. DNA isolated from C57BL/6 and the MSM strain originating from a Japanese wild mouse, *Mus mus molossinus*, were subjected to polymerase chain reaction (PCR). Analysis of the products by electrophoresis on a polyacrylamide gel exhibited a simple sequence length variation between them. A segregation analysis was then carried out for 131 intersubspecific backcross mice obtained by mating (C57BL/6 × MSM) F<sub>1</sub> to MSM males. The strain distribution pattern was compared to that of microsatellite loci that had been determined by published markers (Dietrich et al., 1994). Fig. 4 summarizes the results of typing backcross mice. A clear linkage of the *Dbx2* locus was found with two markers, D15Mit15



(*HoxC8*) and D15Mit40. From this haplotype analysis, I concluded that the most likely order of loci on chromosome 15 was cen. .D15Mit15-0.8 cM-D15Mit40-6.1 cM-*Dbx2*. .telo. Since *Dbx* has been mapped to mouse chromosome 7 (Johnson et al., 1992), *Dbx* and *Dbx2* do not form a gene cluster.

### Expression of *Dbx* and *Dbx2* in the embryonic CNS

The spatio-temporal distribution of *Dbx* and *Dbx2* mRNAs was examined by *in situ* hybridization in embryos from stages older than 6.5 day using *Dbx* or *Dbx2* antisense probes (d and e in Fig. 1). Expression of both genes was detected in the neural tube of embryos of stages later than 9.5 day as shown in embryos hybridized in whole mount (Fig. 5). Remarkably, both *Dbx* and *Dbx2* were expressed in a zone separating alar and basal plates along the length of the spinal cord and rhombencephalon. Expression of *Dbx2* occurred at a lower level and in a broader band than *Dbx* (Fig. 5). *Dbx* expression was also observed in the brain, most conspicuously in the mammillary areas, dorsal thalamus to pretectum, and midbrain.

The regional specificity of *Dbx/Dbx2* expression in the brain was more closely examined in 10.5-15.5 day embryos by *in situ* hybridization of serial sections through sagittal, transverse and frontal planes. Representative examples of *in situ* hybridization data are shown in Figs. 6-8 and summarized as a scheme in Fig. 12. The pattern of expression of these genes did not substantially change during this period. The expression of *Dbx* and *Dbx2* in the brain was restricted to the ventricular zone except for that of *Dbx* in the septum of the telencephalon to be described below.

#### (1) Expression in the telencephalon



In the telencephalon, *Dbx* expression could be detected in the caudal ganglionic eminence (Fig. 7E) and septum (Fig. 6A), while *Dbx2* was not detectable. *Dbx* expression in the septum was detected in cells out of the ventricular zone.

## (2) Expression in the diencephalon

In the diencephalon, *Dbx* was expressed mainly in three domains rostral to the dorsal thalamus. One domain covered the anterior entopeduncular area (Fig. 6C, E) and the second domain covered the eminentia thalami (Fig. 7E). The third domain covered the optic chiasma, basal zone of the hypothalamus, mammillary areas (including the retromammillary area) and zona limitans intrathalamica (the boundary separating the dorsal and ventral thalamus) (Figs. 6A, C, E and 7A). Out of these domains, *Dbx*-positive cells scattered among negative cells in the alar zone of the hypothalamus and caudal region of the anterior preoptic area (Figs. 6C, 7A and 12A). In the same diencephalic subdivision *Dbx2* was expressed in a short band located at the alar-basal boundary, from caudal region of hypothalamus to rostral region of the ventral thalamus (arrows in Figs. 6D, 7B, F and 8B, D-F).

Caudal to the zona limitans intrathalamica, *Dbx* expression in the alar zone started near the rostral boundary of the dorsal thalamus and epithalamus, and was extended through the middle of the pretectum (Fig. 6C). In the caudal half of the pretectum, *Dbx* expression was confined to the boundary between alar and basal zones (Figs. 6C). *Dbx2* was expressed in a continuous band along the alar-basal boundary spanning the dorsal thalamus and the pretectum (Figs. 6D, 7B and 8A, C, E, F). It was noted that expression of both *Dbx* and *Dbx2* increased in the dorsal thalamus away from the zona limitans intrathalamica.

## (3) Expression in the mesencephalon



In the mesencephalon, *Dbx* was strongly expressed in the dorso-lateral wall, continuously from the pretectum and to the rhombencephalic isthmus (Fig. 6A, C, E). Very weak expression of *Dbx2* could also be seen throughout the dorso-lateral wall overlapping with that of *Dbx* (Figs. 6B, D and 10A). In addition, *Dbx2* was expressed at the boundary between the dorso-lateral wall and the basal zone in its rostral half (arrowhead in Fig. 6D).

In the rhombencephalic isthmus, *Dbx* was expressed at a high level while *Dbx2* was not detected (Fig. 6C, D).

#### (4) Expression in the rhombencephalon and spinal cord

Along the length of the neural tube posterior to the rhombencephalic isthmus, the expression of *Dbx* and *Dbx2* was detected as a bilateral pair of narrow bands at the boundary between the basal and alar plates (Figs. 5, 6, 7C-F, 8C, 9).

In the spinal cord, expression of *Dbx* was restricted to the boundary between the alar and basal plates (Fig. 5A and 9C, D). Expression of *Dbx2* was similar to *Dbx* in its distribution, but broader, extending out from both sides of the *Dbx*-expressing band and with less defined borders (Fig. 5B and 9E, F). In the rhombencephalon, expression of both genes was restricted to the boundary of the dorso-ventral zones in a manner similar to that seen in the spinal cord (Fig. 5, 7C-F, and 8C).

At early stages when lateral plates of the spinal cord still consisted of neuroepithelial germinal cells (9.5-10.5 day), expression of *Dbx* and *Dbx2* can be detected throughout the entire thickness of the plates (Fig. 9C, E). At later stages when neural differentiation initiates in the intermediate zone, expression of both genes become confined to the ventricular zone (Fig. 9D, F). But regardless of the developmental stage, the dorso-ventral width of the *Dbx/Dbx2* expressing bands



remained fairly the same. Expression of *Dbx2* appears to increase with the development of the embryo as judged by the increase in intensity of the hybridization signal (Fig. 9E, F).

#### Expression of *Dbx2* outside of the CNS

Although expression of *Dbx* was confined to the CNS, *Dbx2* was also expressed in mesenchymal components of the limb buds and tooth germs.

##### (1) Expression in the limb buds

In the limb bud mesenchyme, a significant level of *Dbx2* expression occurred after 11.5 day (Fig. 10A-C). High expression appears localized to the internal mesenchymal cells which later participate in chondrogenesis. After 13.5 day the expression was localized to the cells surrounding the precartilaginous condensation of the digits (Fig. 10D, E).

##### (2) Expression in the tooth germs

In the tooth germs, *Dbx2* was expressed in the mesenchymal cells surrounding the enamel organ. Data of 13.5 and 15.5 day embryos are shown in Fig. 11. The *Dbx2*-positive cells included both dental papilla and follicle cells, but not the cells immediately adjacent to the internal enamel epithelia of the enamel organ and the cells surrounding the dental ledge. The expression pattern did not substantially change during this period.



## Discussion

### *Dbx* gene family

I have described the gene organization and expression of two new mouse homeobox genes which compose the *Dbx* family. *Dbx* and *Dbx2* have similar homeodomain sequences and exon-intron organizations. *Dbx* cDNA was cloned independently by Lu et al. (1992) and its gene organization reported (Lu et al. 1994), which are in general agreement with my results.

The homeodomain and C-proximal flanking amino acid sequences are very similar between *Dbx* and *Dbx2*, but the sequences of other regions are quite divergent. *Dbx* and *Dbx2* likely can recognize analogous nucleotide sequences, but may differ in their action as transcriptional regulators. Proximal to the C terminus of *Dbx* protein, there is a cluster of acidic residues often present in transcriptional activators (Sigler, 1988), but this motif is absent in *Dbx2*.

Comparison of the homeodomain amino acid sequences indicated that the *Dbx* family is related, but apparently distinct from the *H2.0* family (Fig. 3). 63-68% similarity was found between the *Dbx* homeodomain and the homeodomains of the *H2.0* family: *H2.0* (Barad et al., 1988), *Hlx* (Allen et al., 1991) and *AHox1* (Saiga et al., 1991). Invertebrate members for the *Dbx* family have not yet been reported.

### Expression of *Dbx* and *Dbx2* in the embryonic brain

*Dbx* and *Dbx2* are expressed in the embryonic brain with regional specificities as summarized in Fig. 12A, B. Expression of *Dbx* is mapped in various regions of the diencephalon with boundaries largely consistent with the neuromeric model of the embryonic brain of the mouse proposed by Bulfone et al. (1993), by Puelles and Rubenstein



(1993) and by Rubenstein et al. (1994). *Dbx* expression in the brain had also been reported by Lu et al. (1992 and 1994), but not in great detail. *Dbx2* expression in the diencephalon is limited to the alar-basal boundaries of the caudal hypothalamus to the region rostral to the ventral thalamus, dorsal thalamus and pretectum. In the midbrain, expression of *Dbx* and *Dbx2* largely overlaps along the dorso-lateral wall.

It should be noted that expression of the *Dbx* genes emphasizes the separation of the alar and basal compartments. It is also remarkable that *Dbx* expression in the alar compartment of the pretectum continuous from the dorsal thalamus terminates in the middle of the pretectum with clear demarcation. This indicates that pretectum may be divided into rostral and caudal components as proposed by Figdor and Stern (1993). Thus, the *Dbx* genes provide two important genetic landmarks to the generic map of the brain.

#### Expression in the spinal cord

Along the length of the neural tube posterior to the rhombencephalic isthmus, both *Dbx* and *Dbx2* are expressed at the boundary of the alar and basal plates which later develop to sensory and motor columns, respectively (Fig. 12C, D). It is important to note that these genes are expressed only in mitotically active cells of the ventricular zone in these regions and that these genes encode putative transcriptional regulators. It is possible that the *Dbx* family plays a role in specifying a small population of neural cells at the boundary between the columns, a region classically called the sulcus limitans. Expression of these genes overlaps, but the expression of *Dbx2* is broader in a dorso-ventral direction. Therefore, the cells expressing both *Dbx* and *Dbx2* are flanked by cells expressing only *Dbx2*. The overlapping



expression of the genes may indicate functional redundancy, but I prefer the view that the products of these genes have different transcriptional regulational activities, as suggested by the high divergence outside of the homeodomain.

It is interesting to speculate a possible function for the sulcus limitans cells in relation to expression of *Dbx* genes. It is known that the differentiation of motor neurons depends on inducing signals from the notochord and floor plate (Placzek et al., 1991; Yamada et al., 1991). Cells expressing *Dbx* genes may acquire a capacity to halt the signal from the ventral region which otherwise induces motor neurons throughout the spinal cord. Altman and Bayer (1984) have proposed that the region of the sulcus limitans (called the intermediate plate) is a source of contra- and ipsilaterally projecting relay neurons. The *Dbx* family may contribute to the determination of cell fates of these neurons. In either case, it is interesting to note that a certain class of cadherins is expressed in ventricular zone cells of the sulcus limitans similar to the *Dbx* family (M. Takeichi, personal communication). It is an intriguing possibility that the *Dbx* family of genes may regulate cadherin expression, which modifies cell interactions and consequently alters fate of the neuroepithelial cells.

#### Expression of homologues in other animal species

Zebrafish *hlx-1*, a putative homologue of *Dbx*, shows an expression pattern similar to *Dbx* in the CNS (Fjose et al., 1994). However, prior to expression in the CNS, *hlx-1* is expressed in an area of primitive mesoderm (Fjose et al., 1994) which was not observed in the mouse embryo.

Chicken *CHoxE*, the putative chicken homologue of *Dbx2*, has been reported to be expressed in the developing neural tube posterior to the



rhombencephalic isthmus almost identically to *Dbx2*. But there is no description of more anterior expression or expression in the other organs (Rangini et al., 1991).

### Complementary expression territories with other homeobox genes

The proposed neuromere map of the CNS (Bulfone et al., 1993; Puellas and Rubenstein, 1993; Rubenstein et al., 1994) is based on the expression patterns of various homeobox genes. Examination of the expression of such genes indicated that some are expressed in regions of brain complementary to *Dbx/Dbx2* expression. For instance, *Pax-6* is expressed roughly complementary to *Dbx* in the diencephalon (Walther and Gruss, 1991; Puellas and Rubenstein, 1993). Caudal to rhombomere 6 of the rhombencephalon and spinal cord, *Gbx-2* is expressed both in alar and basal plates, but not in the sulcus limitans (Bulfone et al., 1993), showing a complementarity to *Dbx* family expression. These observations may suggest that characteristics of individual neuromeres are defined by multiple superpositions of dichotomous choice of gene expression. It is interesting to see if complementary pairs of transcription factor genes are generally found with boundaries which conform to the model of the CNS.

### Expression in the limb buds and tooth germs

Both in the limb buds and tooth germs, *Dbx2* is expressed in undifferentiated mesenchymal cells. In the limb buds, *Dbx2* seems to be expressed in the cells prior to participate in the precartilaginous condensation. In the tooth germs, *Dbx2* is expressed in the mesenchymal cells surrounding the enamel organ, but not in the cells immediately adjacent to the internal enamel epithelia which may be



thrombocytopenic purpura (TTP) is identical to *Dbx2*. But there is no description of more recent expression or expression in the other organs (Kang et al., 1995).

### Complementary expression territories with other homeobox genes

The proposed schematic map of the CNS (Hirata et al., 1997) Puelles and Rubenstein, 1993; Rubenstein et al., 1993) is based on the expression patterns of various homeobox genes. Expression of the expression of such genes indicates that some are expressed in regions of brain complementary to *Dbx2* expression. For instance, *Pax6* is expressed mostly complementarily to *Dbx2* in the diencephalon (Walter and Gross, 1997; Puelles and Rubenstein, 1993). *Cxcl-2* is expressed both in the rhombencephalon and spinal cord. *Dbx2* is expressed both in the star and basal plates, but not in the telencephalon (Hirata et al., 1997), showing a complementarity to *Dbx2* expression. These observations may suggest that characteristics of individual neurons are defined by multiple responsiveness of dichotomous choice of gene expression. It is interesting to see if complementary pairs of transcription factor genes are generally found with boundaries which conform to the model of the CNS.

Expression in the limb buds and tooth germs  
Both in the limb buds and tooth germs, *Dbx2* is expressed in undifferentiated mesenchymal cells. In the limb buds, *Dbx2* seems to be expressed in the cells prior to participate in the premitochondrial condensation. In the tooth germs, *Dbx2* is expressed in the mesenchymal cells surrounding the enamel organ, but not in the cells immediately adjacent to the internal enamel epithelium which may be

undergoing differentiation into odontoblasts. Therefore, in the mesenchymal cells of these organs, *Dbx2* seems to be expressed transiently immediately before the start of differentiation, which may suggest that *Dbx2* is playing a role in determination of these cells. This is also consistent with the observation in the CNS that *Dbx2* is exclusively expressed in the undifferentiated cells of the ventricular zone.

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# Figures



Fig. 1. Structure of *Dbx* (A) and *Dbx2* (B) genes. Exon-intron organization maps of genomic clones of *Dbx* (g164) and *Dbx2* (gS4), in comparison with cDNA clones shown below (c164, cS4). Wide boxes indicate the protein coding sequences, open boxes the homeoboxes, and narrow boxes the 3' untranslated regions. a to f indicate probes used for phage library screenig (a, c and f), genomic Southern hybridization (b) and *in situ* hybridization (d and e). BB in the fourth exon of *Dbx* indicates twice repeated *Bam*HI sites separated by a nucleotide between their recognition sequences. Restriction sites, B, *Bam*HI; E, *Eco*RI; H, *Hind*III.

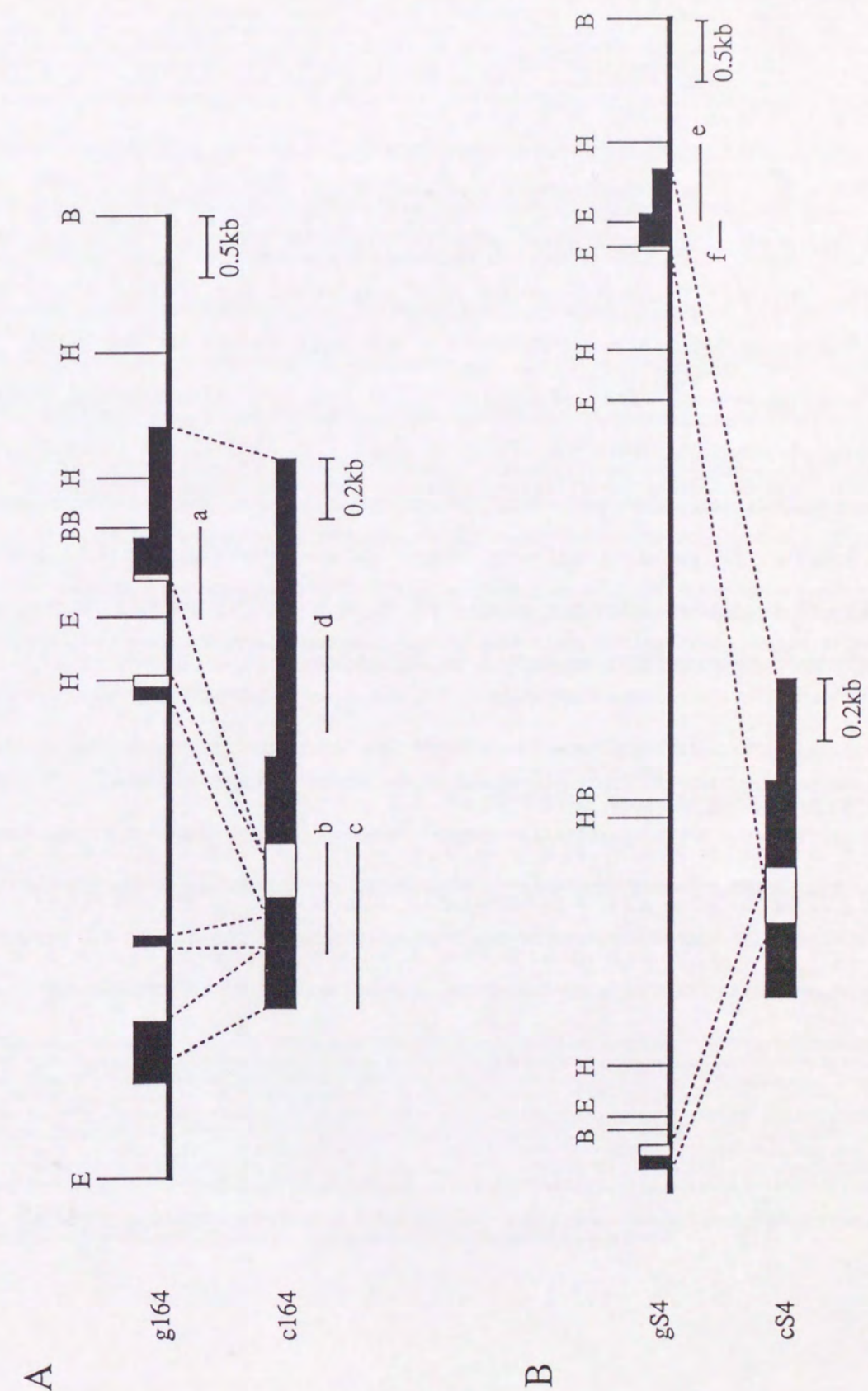




Fig. 2. Nucleotide sequences of *Dbx* (A) and *Dbx2* (B) genes. Nucleotides are numbered arbitrary just to indicate the positions in the Figure. Predicted amino acid sequences are indicated below the nucleotide sequences with residue numbers shown to the right. The homeodomains are shaded. The (CA)<sub>n</sub> repeat, the regions complementary to the PCR primer set used for chromosomal mapping and the polyadenylation signal are underlined. Arrows indicate the putative splicing sites. Solid arrowhead in (A) indicates the 5' end of c164. Sequences of 5' to [ in (B) are cDNA sequence. Open triangles indicate the poly A addition sites.

[illegible]



# B

CACGCACGCACCCCGCCCCACGCCAGCGCCGGTCTGAGTGTCCGCAGCTGCGGCCACTGCCTGCCAGCCAGTGCCTCAAGCT 90  
T H A P P P P R P A P G P E C P Q L R P L P A S P V P L K L 30

GTGCCCCGGCGACCTTTGGTGTGCGCTGGGCCTTCCAGATGCCCCCTGGCCGGGCTCCGGGGAGCGGGACAGTGCCTTCCAGCCCTC 180  
C P A G P F G V R W A F Q M P P G R A P G E R D S A F Q P S 60

AGCCCCAG.....[TGCAGGTCAACGGATCATCATTTTTTGGGTTTCAAGGAAGTCCAGCACTCAGATAGCTTA 248  
A P G 63

AGCAACAGAGAACAGCTGCCACCGTGTGAAGTATGAGATACCAGTGTCTGAGAGTCCGAGGGCTTCCCCTCTCTAACCTGTGCCCTT 338

TTGTATTCTTGTCTCTGCCAGGAGAAGAACACGGGCTGCCTTTGCTGACCCAGGACTCAAATTCCAAAGCTCGCAGGGGATTTACGA 428  
E E H G L P L L T Q D S N S K A R R G I L R 85

AGAGCTGTGTTCTCAGAGGAGCAGAGAAAGGCTCTGGAGAAAATGTTTCAGAAGCAGAAATACATCAGCAAAACAGACCGAAGGAAACTC 518  
R A V F S E E Q R K A L E K M F Q K Q K Y I S K T D R R K L 115

GCGGTACGCTTGGGACTGAAGGAGTACAGGTACAGTGAAGGAGGATGCATCTAGTCTACTTTCAACAGCAGTGTGAAATTTGTTT 608  
A V S L G L K E S Q 125

TTTTGCCAAATATCTTGTGCCAGGTTGTACCCAAAGTGTCTGAATTTTTCAGTAGGTTGAGGCCCATGGGACAAATCCTATGTGAA 698  
GACAGGATCCACACACGCTGAGGTGGCAAAGCAGACCGCAGAATTC(-6.4kb-) CACATTAACACCCCTGCACATAAACATGTGTGTG 778  
CATATACATACACAGACACCACGTGTGCATCTGCATGTTTCATGCATGCGCACACACAAATACATGCACACACACACATACACAC 868  
ACACACACACACACACACACACACACACACTCCCCGATGCAATCCAATTAATCAGCCAAATCAACACACATAGGTGCTCTACTA 958  
GTCAATTTTCTTGAATAATGTATCAGAATAGAGCAAAATATAATGATGTTTAAAGTATTAAACTAAGTTAATTTCAACAAGCCACA 1048  
GAAATACAGAGTGAATCAGTGTCTACACTCATCTACTTAGAAAAGTGCCTGTGGTGTGCGGGTGTGTTTCTCAAGTGTCTTTGAAGGA 1138  
ACCCACGGGAAAAGCCACAGGGAGTCTCTAGTTAGACTTCCAGAGTAGGAGATTCTGCAGTAAGTATAGTCGAAGCAGTTAGCAGTTGGG 1228  
GATGTTGAAGAGGGATCGGAATATGCAGTTACCCAGCATATATGAGGCCAAAGTCAAAGAAAATAAGGAAAACACTCAAGTGTGTATG 1318

CGGAATAAGGACTTCTTTCCTTATTATTTTATCATTAGGTGAAGATTGGTTCCAGAACCAGGATGAATGGCGAATTCCAAAGAAA 1408  
V K I W F Q N R R M K W R N S K E K 143

AGGAAGTACTCTCCAGTAGGTGTCTCCAAGAAGTGAAGCTTCAGGAAGACAGGCTGGCACGGCCTGCCGTGGGCTGTCTCCGAGTGCC 1498  
E V L S S R C L Q E V S L Q E D R L A R P A V G C P P Q C P 173

CGTCAATATGGGAAGTCTCCAGCCACACTCAAGTCCAAGCTGGAGGAGGAGACTCCAGAATCTGCAGAAAGACTGACCCAGGAGAATT 1588  
S I W E V S Q P H S S P S W R E E T P E S A E R L T Q E N S 203

CAGGGGTCTGGAAGCAGATTCACTCCGAGGTACCTTGTATCTGTGTCTGAGAAGGGACCTAGAGACAAGCATGGACTCCAGAGCACCA 1678  
G V L E A D S L R G T L Y L C P E K G P R D K H G L Q S T I 233

TTTGACGGGAACATACTCCGTGTACATCTAAAGAACCCTTAGCCAGTAACACTTGGATTAAAGCCAGTTAGATTGTGCCTCAAACTGC 1768

CTTAAACTAATACCTTGGCATGATGCCTGAGCTGTTGCCTAAGGAGAGCCACTCTACTGTTTCTTATTAGCCCTAGCCTAAGGCTCAAT 1858  
GTATATATGGAATAGAACCTTCCACAAAGTGTGATAAGACTGAAAGAAGGCCAGGATCAGCCCCAGAGCCTGTTCTGAATATGTGTACA 1948

TATTCGTGTGTCTGTGTGTTGTATGTATATGCTCATTAAATACATCATCAGAAATTAGTCTCTCAAGTTTATATGGAACAAATTCAT 2038  
TTCAATTTGTATAAGTAAAAAAGGACCAACGAGCAATATCATTTTCTAATAGTGAATCTGGGCTT 2111



Fig. 3. Amino acid sequences of the homeodomains of *Dbx* and *Dbx2* in comparison with those of *CHoxE* (Rangini et al., 1991), *hlx-1* (Fjose et al., 1994), *Hlx* (Allen et al.), *H2.0* (Barad et al., 1988), *AHox1* (Saiga et al., 1991) and *Antp* (Scott and Weiner, 1984). Similarity score of each homeodomain sequence relative to *Dbx* is shown on the right together with the animal species from which the gene was derived. Dashes indicate the residues identical to *Dbx*.

|              |             |           |           |          |           |          |           |            |         |           |       |     |           |
|--------------|-------------|-----------|-----------|----------|-----------|----------|-----------|------------|---------|-----------|-------|-----|-----------|
| <i>Dbx</i>   | GMLRRVFS    | DVQRKALE  | KTFFQKQKY | ISKPD    | RKKL      | ASKLGL   | KDSQ      | VKIWF      | QNR     | RMKWR     | NSK   |     | mouse     |
| <i>Dbx2</i>  | -I-----     | -EE-----  | -M-----   | -T-----  | -R-----   | -VS----- | -E-----   | -----      | -----   | -----     | ----- | 85% | mouse     |
| <i>CHoxE</i> | -I-----     | -ED-----  | -M-----   | -T-----  | -IN-----  | -E-----  | -----     | -----      | -----   | -----     | ----- | 87% | chick     |
| <i>hlx-1</i> | -----       | -----     | -M-----   | -----    | -A-----   | -----    | -----     | -----      | -----   | -----     | ----- | 97% | zebrafish |
| <i>Hlx</i>   | RSWS-----   | -NL-----  | -G-----   | -R-EI-   | -VT-----  | -Q-----  | -AM-----  | -T-A-----  | -V----- | -H-----   | ----- | 68% | mouse     |
| <i>H2.0</i>  | RSWS-----   | -NL-----  | -G-----   | -IQ----- | -Q-----   | -T-----  | -R-----   | -AR-N-T-A- | -V----- | -HTR----- | ----- | 65% | fly       |
| <i>AHox1</i> | RKWN-----   | -LM-----  | -RG-----  | -S-----  | -S-----   | -VA----- | -E-R----- | -DA-S-T-A- | -----   | -QEI----- | ----- | 63% | ascidian  |
| <i>Antp</i>  | RKRG-QTYTRY | -TLE----- | -E-HFNR   | -LTRRR   | -IEI-HA-C | -TER-I   | -----     | -----      | -----   | -----     | ----- | 38% | fly       |



Fig. 4. Distribution of the haplotypes for 131 progeny from intersubspecific backcross mice. The loci followed in the cross are indicated with arrows to the partial chromosome 15 linkage map. The filled squares represent the C57BL/6 allele, and the open squares represent the MSM allele. Each column represents the chromosome identified in the progeny. The numbers of the progeny carrying each type of chromosome are listed at the bottom. In the linkage map, more proximal markers mapped previously are indicated in smaller type (Dietrich et al. 1994; Suda et al. 1994).

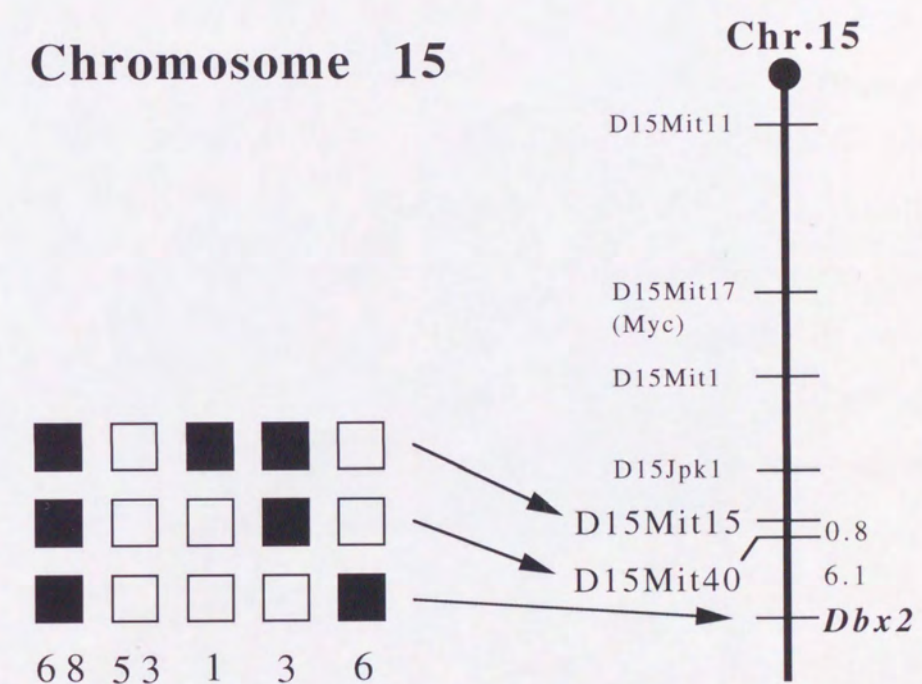




Fig. 5. Whole-mount *in situ* hybridization data of 9.5 day embryos probed with *Dbx* (A) and *Dbx2* (B). The procedure for detection of *Dbx* and *Dbx2* was slightly different as described in Experimental Procedures. The stripes of *Dbx* and *Dbx2* expression along the length of rhombencephalon to spinal cord are indicated by arrowheads. In (A), three conspicuous sites of *Dbx* expression are marked: me, mesencephalon; d, a complex of areas from dorsal thalamus to rostral pretectum; ma, mammillary to retromammillary areas. In (B), deposit of the stain in structures other than the CNS was not reproducible. Scale bar, 0.5 mm.

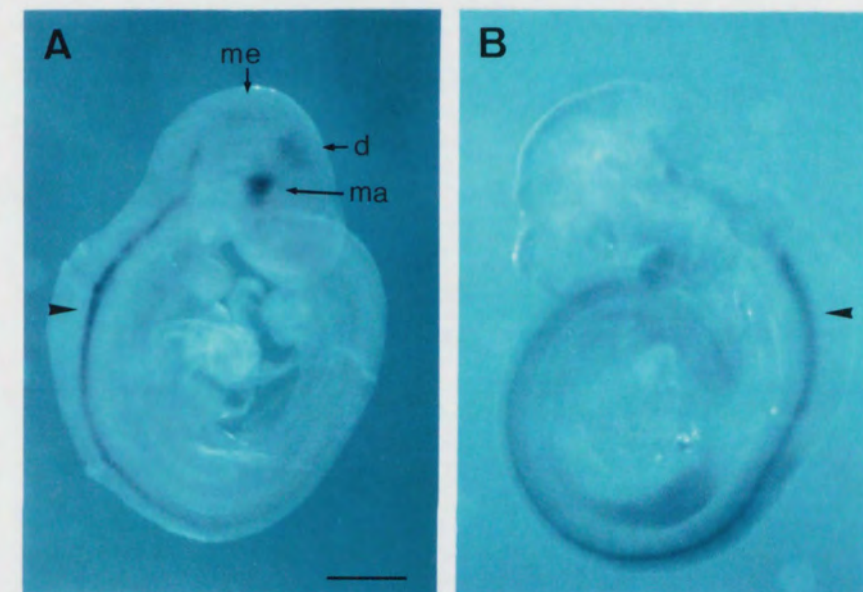




Fig. 6. Expression pattern of *Dbx* (A, C and E) and *Dbx2* (B and D) in 11.5 day embryo in sagittal sections. *In situ* hybridization data are shown. (A) and (B), (C) and (D) are pairs of adjacent sagittal sections. (E) is a medial section parallel to (C). (F) shows hybridization with sense probe. An arrowhead in (D) emphasizes the hybridization signal at the boundary between the dorso-ventral wall and the basal zone of the mesencephalon which may escape from attention. An arrow in (D) indicates the expression of *Dbx2* in the caudal region of the hypothalamus. Lines with labels AB, CD and EF indicate the positions and orientations of transverse and frontal sections in Fig. 7. Abbreviations: aep, anterior entopeduncular area; bh, basal zone of the hypothalamus; cpt, caudal pretectum; dt, dorsal thalamus; h, hypothalamus; is, rhombencephalic isthmus; m, mesencephalon; ma, mammillary area; oc, optic chiasma; poa, anterior preoptic area; pt, pretectum; rh, rhombencephalon; rpt, rostral pretectum; sc, spinal cord; se, septum; vt, ventral thalamus; zi, zona limitans intrathalamica. Scale bars, 1 mm.

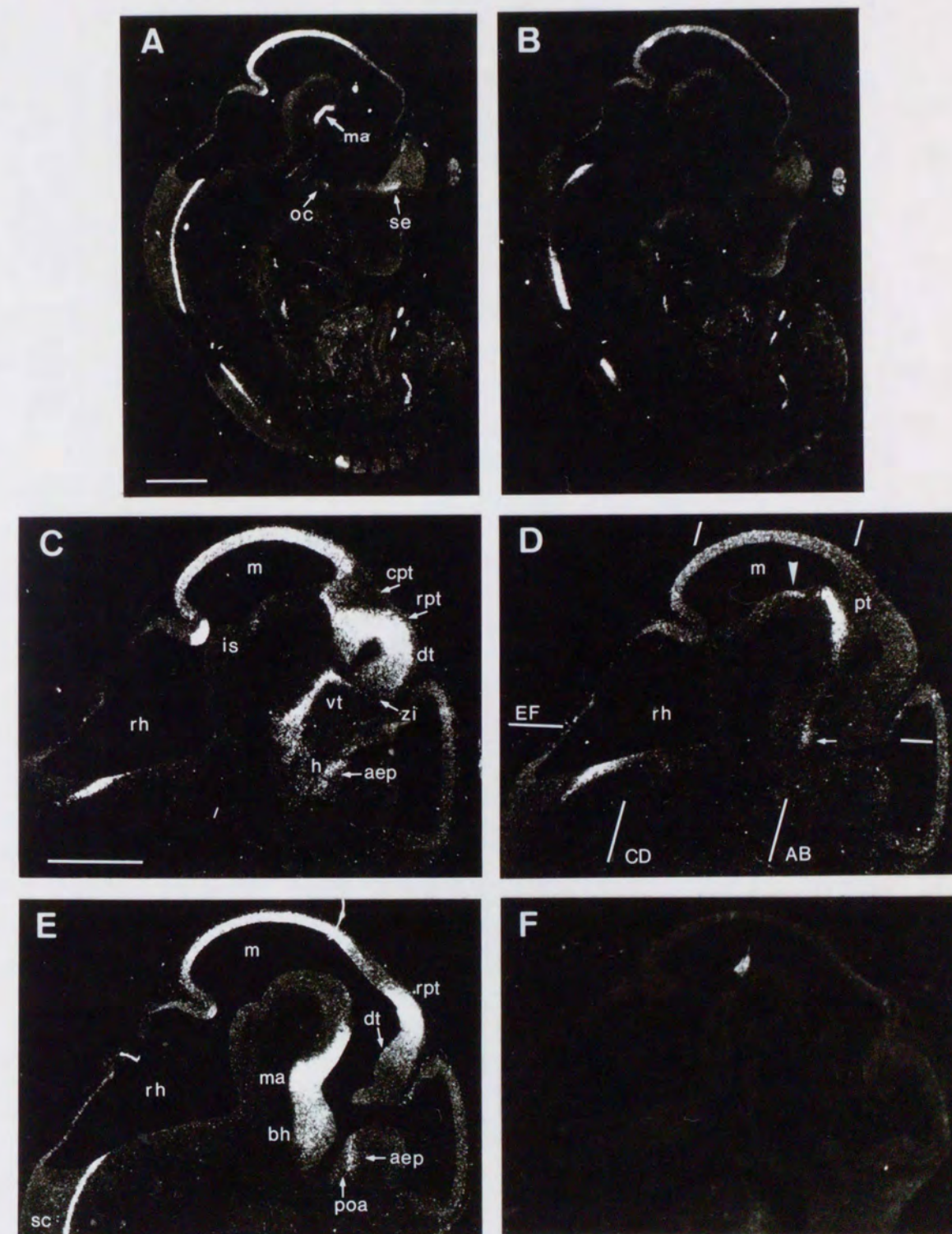




Fig. 7. Frontal (A-D) and transverse (E, F) sections showing expression of *Dbx* (A, C and E) and *Dbx2* (B, D and F) in 11.5 day embryonic brain. (A) and (B), (C) and (D), (E) and (F) are adjacent pairs of sections. Arrows in (B) and (F) indicates the expression of *Dbx2* in the caudal region of the hypothalamus. The positions and orientations of sections are indicated in Fig. 6. Abbreviations: cge, caudal ganglionic eminence; emt, eminentia thalami; otherwise the same as in Fig. 6. Scale bars, 1 mm. Scales for (E) and (F) are the same as (A).

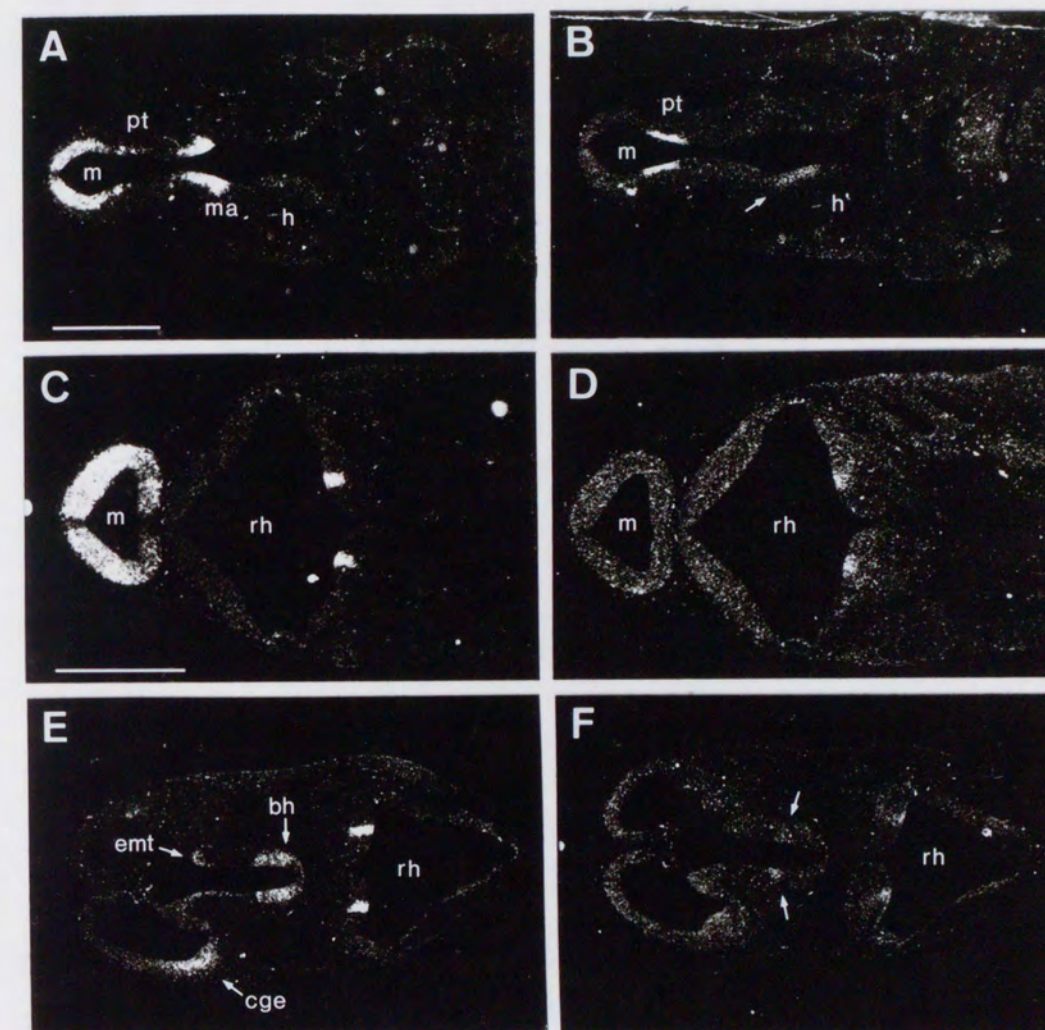




Fig. 8. Expression pattern of *Dbx2* in the embryonic brain of the stages other than 11.5 day. (A) and (B) are serial frontal sections of 10.5 day embryonic brain. (C) and (D) are serial transverse sections of 12.5 day embryonic brain. (E) and (F) are frontal sections of 13.5 day and 15.5 day embryonic brains, respectively. Arrows in (B), (D), (E) and (F) indicates the expression of *Dbx2* in the caudal region of the hypothalamus. Arrowheads in (E) and (F) indicates the expression of *Dbx2* in the pretectal area. Signal in the telencephalon close to the bottom of the figure (D) is artifact of the section. An area of shining between the telencephalon and diencephalon in (E) is due to hemocytes rather than to a signal. Abberivations are the same as in Figs. 6 and 7. Scale bars: (A), 0.5 mm; (C) and (E), 1 mm. Scale for (F) is the same as (E).

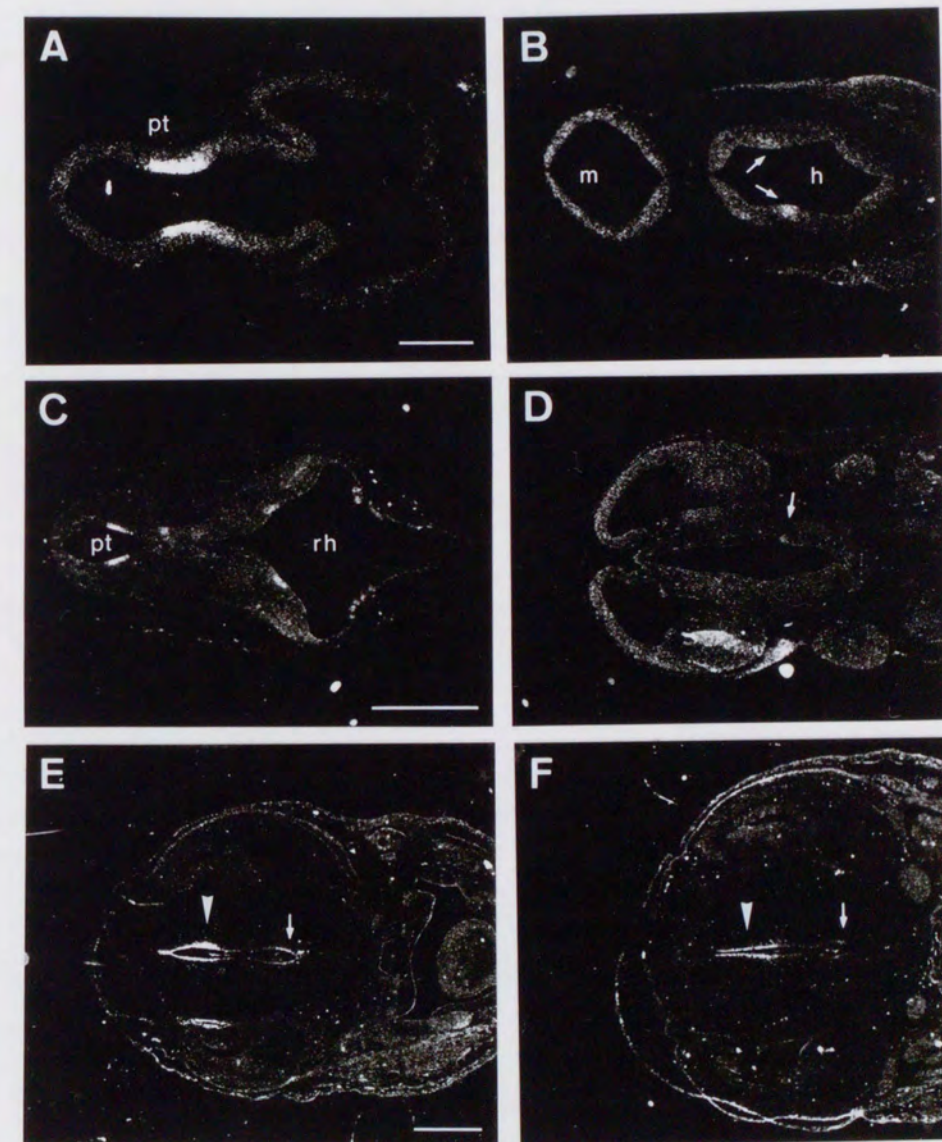




Fig. 9. Comparison of *Dbx* (C and D) and *Dbx2* (E and F) expression in the spinal cord using adjacent transverse sections of 10.5 day embryos (A, C, E) and 12.5 day embryos (B, D, F). Sections of 10.5 day embryos are at the hind limb level and those of 12.5 day embryos are at a cervical level. (A) and (B) are phase contrast images of (E) and (F), respectively. Scale bar, 0.2 mm.

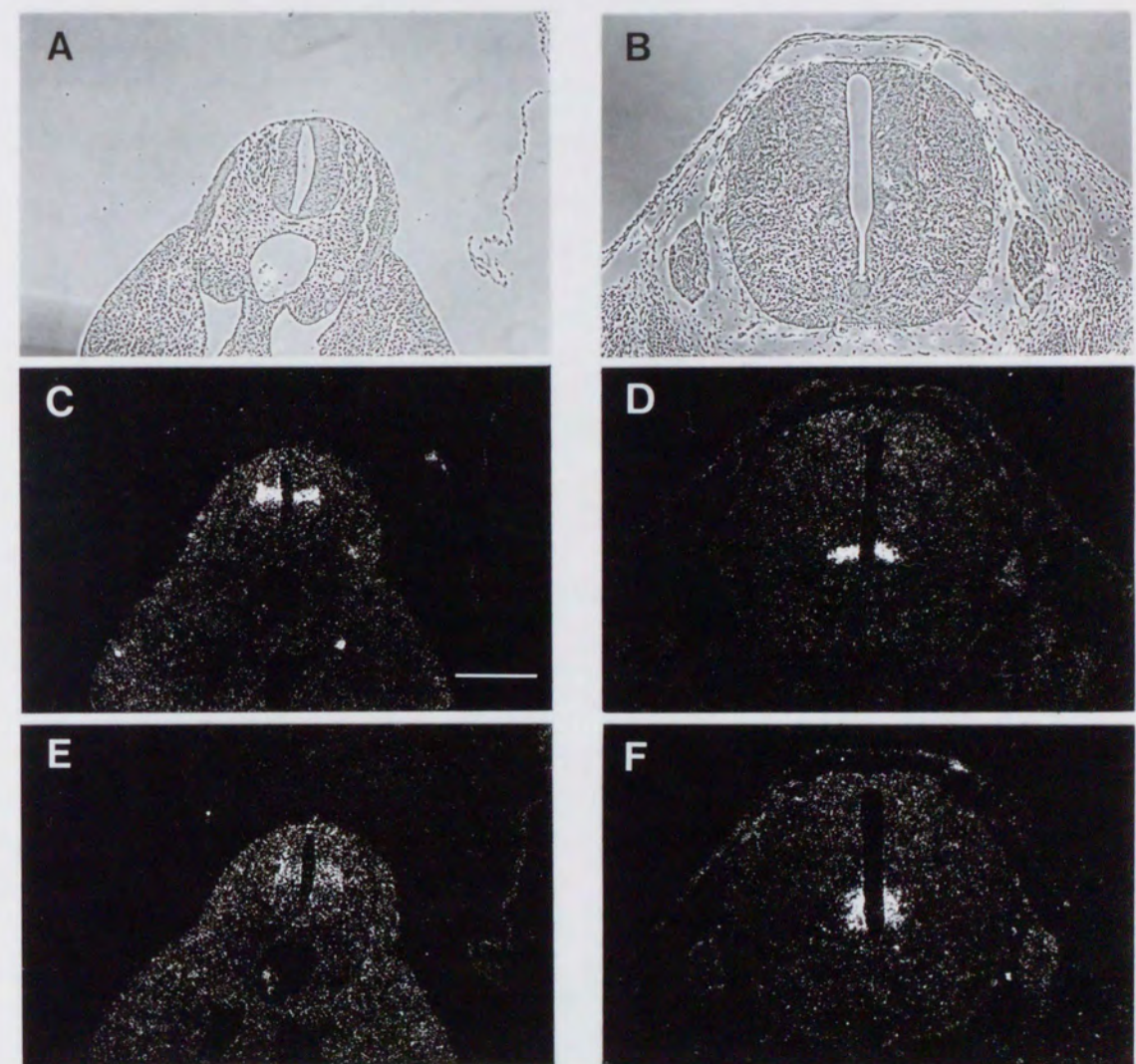




Fig. 10. Expression of *Dbx2* in the limb bud mesenchyme. (A)-(C). A frontal section of 11.5 day embryo at different magnifications showing expression of *Dbx2* in the hind limb bud. Arrows in (A) indicate the hybridization signal in the dorso-lateral wall of the mesencephalon (m), the hind limb bud (hl) and the spinal cord (sc). (B) is a magnification of the hind limb bud in (A). (D) and (E). A transverse section of 13.5 day embryonic fore limb bud at level of digits. Arrows in (D) indicate the hybridization signal. I and V in (F) indicate the digit number. (C) and (E) are phase contrast images of (B) and (D), respectively. Scale bars: (A), 1 mm; (B), 0.2 mm. Scales for (D) and (E) are the same as (B).

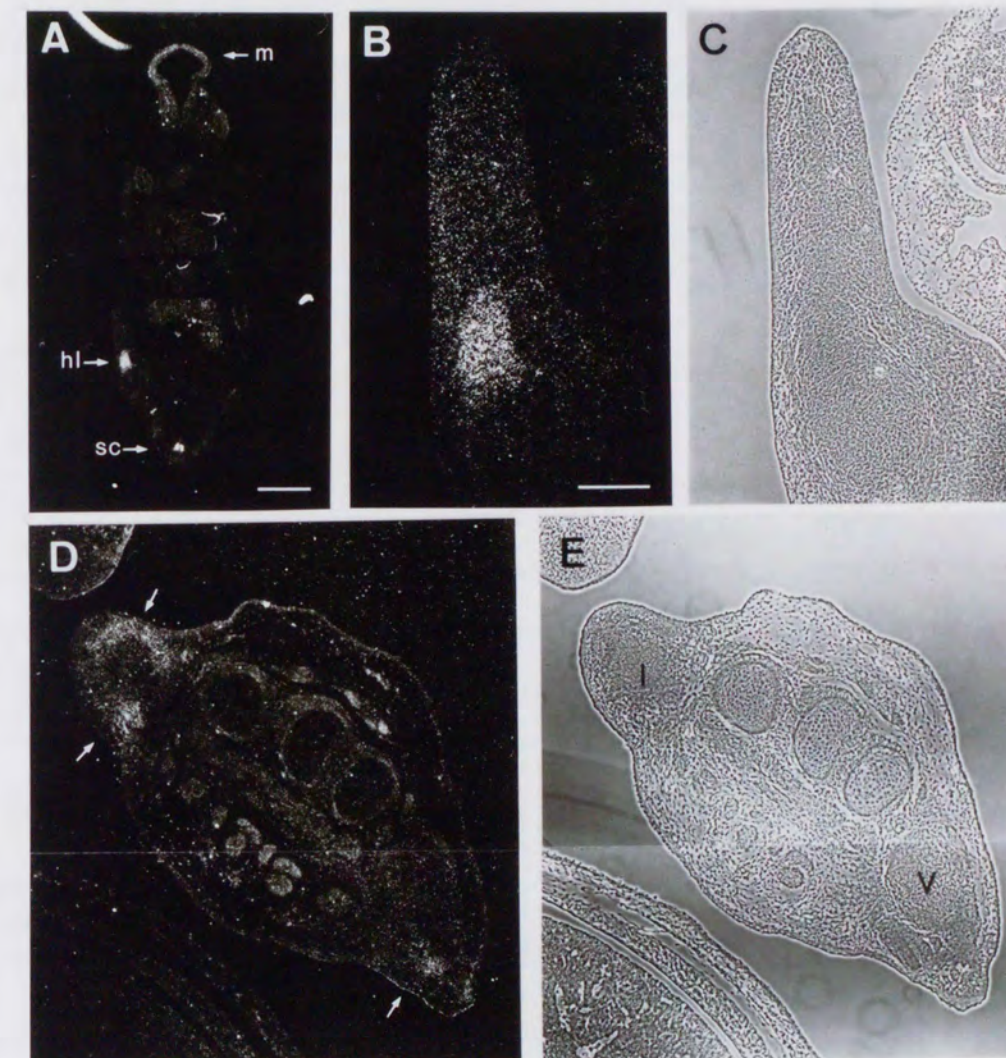




Fig. 11. Expression of *Dbx2* in the tooth germ mesenchyme. (A)-(C) and (D)-(F) are frontal sections of 13.5 day and 15.5 day embryonic head, respectively. (B) and (E) are magnifications of the tooth germs indicated with arrowheads in (A) and (D), respectively. Arrows in (A) and (D) show expression of *Dbx2* in the other tooth germs. (C) and (F) are phase contrast images of (B) and (E), respectively. The region between two arrowheads in (F) is dental ledge. Signal in lens seen in (A) is non-specific. Abbreviations: df, dental follicle; dp, dental papilla; ie, internal enamel epithelia. Scale bars: (A), 1 mm; (B), 0.2 mm.

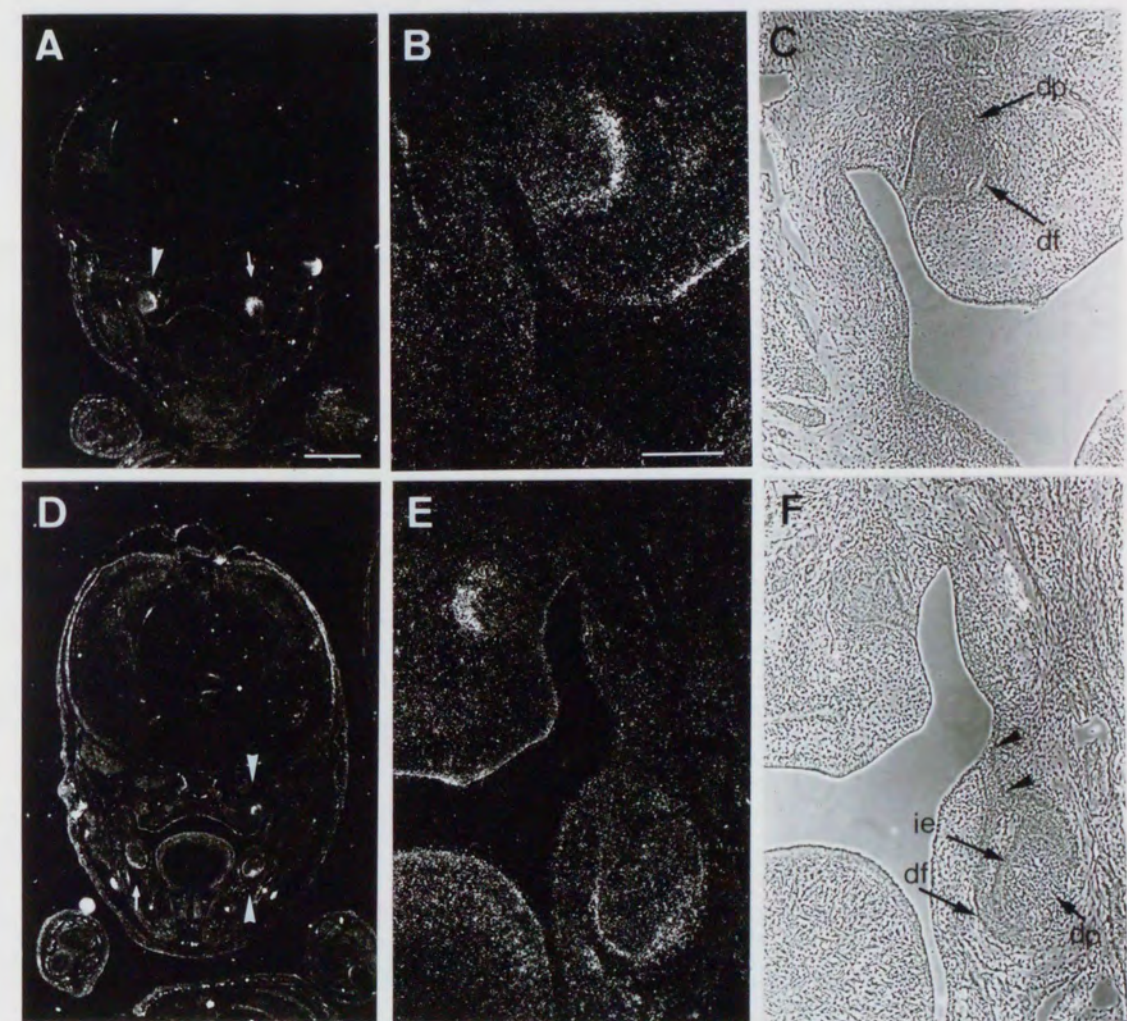




Fig. 12. Schematic illustrations of *Dbx* and *Dbx2* expression patterns. (A) and (B). Expression of *Dbx* and *Dbx2*, respectively, in the brain of 11.5-12.5 day embryo. Gray areas indicate expression domains. The intensity of these paints reflect the relative intensity of hybridization signals in each illustration. Shaded area indicates scattering of *Dbx*-positive cells. (C) and (D). The spinal cord of 9.5-10.5 day embryos (C) and 11.5-12.5 day embryo (D). Filled areas are the regions expressing both *Dbx* and *Dbx2*, and shaded areas the regions expressing only *Dbx2*. Abbreviations: aep, anterior entopeduncular area; ap, alar plate; bh, basal zone of the hypothalamus; bp, basal plate; cge, caudal ganglionic eminence; cpt, caudal pretectum; dt, dorsal thalamus; emt, eminentia thalami; et, epithalamus; fp, floor plate; h, hypothalamus; is, rhombencephalic isthmus; lge, lateral ganglionic eminence; m, mesencephalon; ma, mammillary area; mge, medial ganglionic eminence; oc, optic chiasma; os, optic stalk; poa, anterior preoptic area; r1-r8, rhombomere 1-8; rm, retromammillary area; rp, roof plate; rpt, rostral pretectum; sc, spinal cord; se, septum; vt, ventral thalamus; vz, ventricular zone; zi, zona limitans intrathalamica.

