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Mouse *Cdx-1* expression during gastrulation

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SUMMARY

We describe the expression pattern of the mouse *Cdx-1* gene during early development, examined by both RNA and protein analyses. *Cdx-1* expression began with the onset of the head process formation (day 7.5) in ectodermal and mesodermal cells of the primitive streak. Expression extended initially to the middle of the prospective hindbrain and subsequently regressed caudad to the spinal cord level by day 9.5. The mesoderm-specific expression was detected in the first somites and could be followed during their differentiation to the myotome of the dorsal somitic edge by day 12. The developing limb buds and the mesonephros exhibited expression up to day 12. No signal could be detected in notochordal cells and cells of the definitive endoderm. Thus, *Cdx-1* is expressed during gastrulation when anterior-posterior positional values are established along the embryonic axes. Furthermore, the expression correlates with the formation of segmented tissue in the posterior hindbrain, the spinal cord and structures like the mesonephros.

Key words: *Cdx-1*, homeobox, caudal, primitive streak, gastrulation

INTRODUCTION

A common feature of multicellular organisms is the establishment of an anterior-posterior polarity and regionalisation along this axis during development. Cell mixing and cell lineage experiments in vertebrates revealed that the A-P axis is formed during gastrulation (Beddington, 1981; Dale and Slack, 1987; Tam and Beddington, 1987). The primitive streak in mammals and birds, or the blastopore in *Xenopus*, are morphological structures that appear at the onset of gastrulation. These structures are involved in the orderly ingression of cells from the inner layer (epiblast) of an embryo to the inside to become mesoderm and endoderm (for review see Bellairs, 1986). This process initiates a sequential pattern of tissue along the anterior-posterior axis of the embryo and is therefore considered one of the most important events during vertebrate embryogenesis.

Several genes, expressed during various stages of gastrulation, have helped in the elucidation of the molecular basis of this event. Many of them bear a common conserved sequence motif, the homeobox (Scott et al., 1989). The homeobox gene *goosecoid* recently isolated from *Xenopus* (Cho et al., 1991) and mouse (Blum et al., 1992) shows a very restricted expression pattern at the onset of gastrulation, suggesting an involvement in the formation of the primitive streak. Several other homeobox genes also exhibit expression in the primitive streak, such as *Evx-1* (Bastian and Gruss, 1990; Dush and Martin, 1992) and members of the clustered Hox genes, like *Hox-1.6* (Sundin et al., 1990), *Hox-2.9* (Frohman et al., 1990), *Hox-1.5, Hox-3.1* (Breier et al., 1988; Gaunt, 1987), *Hox-1.4, Hox-2.6* and *Hox-4.2* (Gaunt et al., 1989). Their expression patterns show overlapping domains with distinct anterior boundaries along the embryonic axis (Dressler and Gruss, 1989; Duboule and Dollé, 1989; Graham et al., 1989; for review see Shashikant et al., 1991). Several lines of evidence support the notion that Hox genes determine the regional specificity. In particular, experimental alteration of Hox gene expression in transgenic mice (Kessel et al., 1990; Le Mouellic et al., 1992) and by retinoic acid (Kessel and Gruss, 1991) leads to transformations of segment identities.

Another class of genes, expressed during gastrulation, belong to the caudal type homeobox (Duprey et al., 1988; Frumkin et al., 1991). The *Chox-cad* gene (Frumkin et al., 1991) in chicken exhibits expression in the primitive streak and cells of the definitive endoderm. Later in embryogenesis, *CHox-cad* activity is restricted to the epithelial lining of the embryonic gut, suggesting its possible involvement in establishing the endodermal germ layer. The isolation of caudal-type homeobox genes from the dorsal lip of *Xenopus laevis*, namely the *Xcad-1* gene and the *Xcad-2* gene (Blumberg et al., 1991), confirms the gastrulation-specific activity of these genes. Mouse members of the caudal class homeobox genes like the *Cdx-1* gene (Duprey et al., 1988) and the *Cdx-2* gene (James and Kazenwadel, 1991) have been reported. But their expression has been documented only in endodermal cells of the late embryo and the adult intestine, while the gastrulation-specific expression of the *Cdx-1* gene thus far was implied only by its isolation from a day 8.5 p.c. embryonic mouse cDNA library.

In this paper, we report the RNA and protein distribution of the mouse *Cdx-1* gene during gastrulation. The earliest expression is detected in the primitive streak at day 7.5 p.c. and can be therefore correlated with the onset of

the head process formation. Later in development, *Cdx-1* expression is localized to the ectodermal and the mesodermal cells of the primitive streak, the neural tube, the somites and the limb buds. Surprisingly, no early endodermal expression could be detected. Implications of these findings will be discussed.

MATERIALS AND METHODS

In situ hybridization

Single-stranded RNA probes were generated by in vitro transcription of a plasmid clone (pBSI) containing a *Pvu*II-cDNA-fragment encoding the 3′ part of the translated region (without the homeobox). Coding and noncoding strands of these fragment were prepared by using 100 μCi ^{35}S-CTP and T3 or T7 RNA polymerases. Probes were dissolved at a final activity of 5×10^4 cts/minute/μl in hybridization buffer. Embryos for in situ analysis were obtained from natural matings of female NMRI mice and male C57Bl/6 mice. The day of the vaginal plug was designated day 0.5. The RNA in situ analyses were done as described in Kessel and Gruss (1991).

Antibody preparation

Polyclonal rabbit sera were raised against peptides encoding the amino- and carboxy termini of *Cdx-1*. The peptides were made by FMOC-synthesis on a Milligen-Biosearch-9050 peptide synthesizer.

NH_2 terminus: MYVGYVLDKDSPVYPGPARPC
COOH terminus: CPTNAGLLGTPSPVPVKEEFLP

The amino terminus was predicted from the investigated 5′ sequences of *Cdx-1*, which will be described elsewhere. For this peptide, it was necessary to place a cysteine residue at the COOH-end in order to facilitate the coupling reaction. The peptides were coupled on ovalbumin by the use of male-imido-benzoyl-N-hydroxy-succinimid-ester (MBS, Sigma), as described by Green et al. (1982).

Chinchilla rabbits were immunized intramuscularly with 250 μg peptid-conjugate with Freund's complete adjuvant (1:1). Booster immunizations of peptide-conjugate emulsified with incomplete Freund's adjuvant were given 4, 10 and 16 weeks after the first inoculation. Rabbits were bled (30 ml) via the marginal ear vein 10 days after each injection, beginning after the second boost.

Specific antibodies were purified using an antigen-affinity column prepared by coupling the peptide to EAH-Sepharose (Pharmacia) using MBS as the coupling reagent. After elution of the antibodies, they were precipitated with ammonium sulphate, resuspended and dialysed in PBS overnight.

Western blot analysis

Extracts of 208 fibroblast cells (a rat-1 subclone, R. Müller personal comm.) and embryonal carcinoma cells F9 (Strickland and Mahdavi, 1978) were prepared by heating the cells for 5 minutes at 95°C in SDS-sample buffer (Laemmli, 1970). Extract of embryo or tissue was prepared by sonification in SDS-sample buffer, followed by heating to 95°C. 30 μg of extracted protein per lane was loaded on a 10% SDS-polyacrylamide gel. Proteins were transferred electrophoretically onto Immobilon PVDF (Millipore) transfer membranes (Kyhse-Anderson, 1984).

The membrane was then blocked with 5% milk powder in PBS for 1 hour at room temperature and incubated with a 1:100 dilution of the Cdx-1 antisera overnight at 4°C. After four times washing with PBS, the membrane was incubated with a 1:1000 dilution of goat anti-rabbit IgG-peroxidase-conjugate (Dako) for one hour. The same washing as for the first antibody was followed by diaminobenzidine treatment to visualize the protein bands (Harlow and Lane, 1988).

Preparation of mouse embryos for antibody staining

Mouse embryos were staged according to Rugh (1968). Embryos for immunostaining on sections were fixed at day 7.5 to 9.5 p.c. with the deciduae in Bouin's fixative for 2 hours, washed in 70% ethanol and embedded in paraffin. The immunostaining was done as described in Oliver et al. (1988).

Embryos for whole-mount antibody staining were dissected in PBS, fixed in 80% methanol/20% DMSO for 2-12 hours and bleached in 2 parts fixative:1 part 30% H_2O_2 for 2 days. After this, the embryos were washed in methanol and stored at −20°C.

For immunostaining, embryos were rehydrated in TBST (10 mM Tris-HCl, pH 6.5-7.0; 150 mM NaCl; 0.05% Tween-20) 2× for 30 minutes and incubated overnight with a 1:100 dilution of the Cdx-1 antisera in blocking solution (80% newborn calf sera, inactivated; 20% TBST) at 4°C. The embryos were carefully washed 5× for one hour at RT and incubated overnight in a 1:200 dilution of the goat anti-rabbit IgG-peroxidase-conjugated second antibody in blocking solution at 4°C. After the same washing as described for the first antibody, the embryos were stained with developing solution (0.6 mg/ml diaminobenzidine) for 10 to 20 minutes. Colour development was stopped by TBST washing. The embryos were transferred to 100% methanol for 30 minutes and then in clearing solution (2 parts benzylbenzoate:1 part benzyl alcohol).

RESULTS

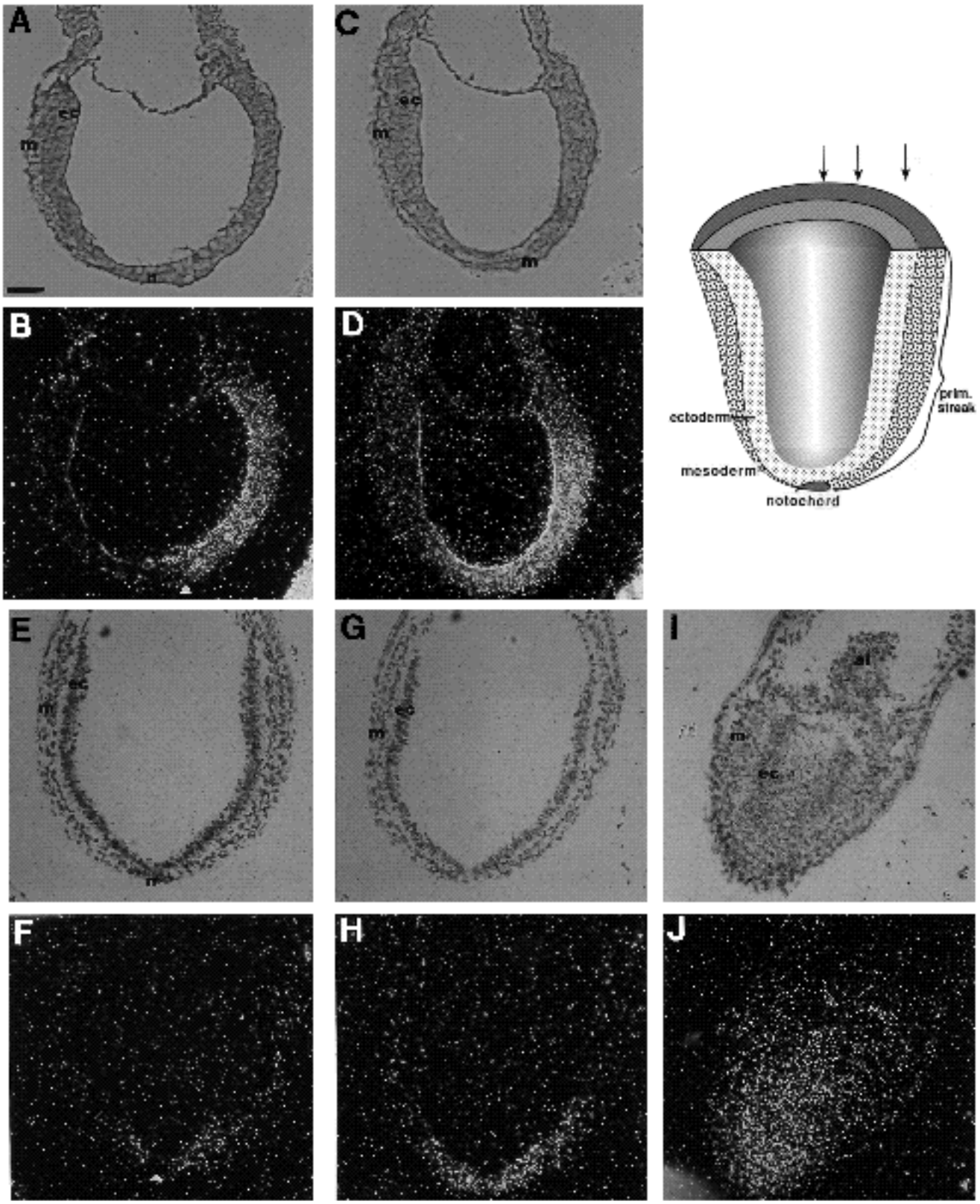
Distribution of *Cdx-1* transcripts during gastrulation

In order to determine the temporal and spatial expression pattern of the *Cdx-1* gene, we examined mouse embryos from day 6.5 to day 12 p.c. by in situ hybridization. We used as a probe a 175 bp *Pvu*II cDNA fragment containing sequences located 3′ of the conserved homeobox.

At early and mid primitive streak stages (day 6.5 p.c. and day 7.0 p.c.), no *Cdx-1* transcript was detected (data not shown). The earliest expression was observed at day 7.5

Fig. 1. In situ hybridization of *Cdx-1* on embryos at day 7.5 p.c. of gestation. Sagittal views are presented with the embryo orientation, anterior to the left, posterior to the right. On the right side is a schematic representation of a midsagittal cut through a 7.5 day p.c. embryo. (A) Midsagittal section showing the early head process formation, visualized by the appearance of the notochordal cells at the tip of the egg cylinder. (B) Dark-field image of A. The arrow indicates the anterior boundary of *Cdx-1* expression in the upper cell layer, which corresponds with the boundary between the notochordal cells and mesodermal cells. (C) Parasagittal section of the same embryo as in A. (D) Dark-field image of C. (E-J) Frontal sections from the anterior to the posterior end of the embryo, the embryonic level is indicated by arrows in the schematical illustration. (E) Section sligthly anterior to the primitive streak presenting the most posterior notochordal cells at the tip of the egg-cylinder. (F) Dark-field image of E. Arrow indicates the area of the notochordal cells. (G) Frontal section at the primitive streak level. (H) Dark-field image of G. (I) Section through the posterior area of the embryo passing the allantois. (J) Dark-field image of I. Bar, 5 μm. Abbreviations: al, allantois; ec, ectoderm; m, mesoderm; n, head process.

p.c. (late streak stage) in ectodermal and mesodermal cells of the primitive streak (Fig. 1A-D). At this time point, the primitive streak is already fully extended and, with the appearance of the node, the process of head process formation begins. Rostral to the node, morphologically distinct notochordal cells appear ventrally at the tip of the egg cylinder (Jurand, 1974). In the area of the primitive streak, *Cdx-1* expression was restricted to the ingressing zone at the anterior end but became progressively broader towards the posterior end of the embryo (Fig. 1E-J). Anterior to the



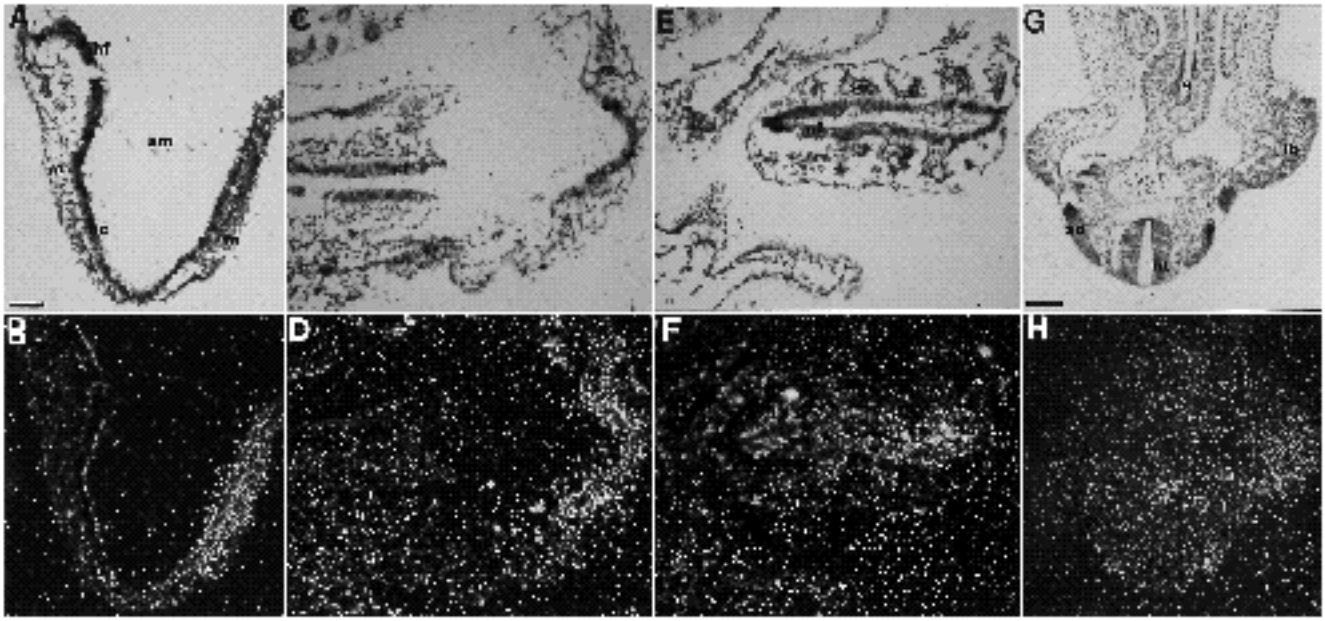


Fig. 2. In situ hybridization analysis of *Cdx-1* at day 8.0 p.c. to day 9.5 p.c. of embryogenesis. The embryo orientation in A-F is anterior to the left and posterior to the right. (A) Sagittal section of a 8.0 day p.c. embryo, no somite condensation is visible at this stage. (B) Dark-field image of A. (C) Transverse section of a 8.5 day p.c. embryo. The section cuts through the anterior neural tube and the posterior primitive streak area. (D) Dark-field image of C. (E) Transverse section of the same embryo as in C, passing through the neural tube and the adjacent somites. (F) Dark-field image of E. (A-F) Same magnification: bar, 5μm. (G) Transverse section of a 9.5 day p.c. embryo. The section shows the area of the forelimb bud and the mid gut structure. Bar, 10 μm. (H) Dark-field image of G. Abbreviations: am, amnion; ec, ectoderm; hf, headfold; g, gut; m, mesoderm; nec, neuroectoderm; nt, neural tube; so, somite.

node, *Cdx-1* expression extended into the neurectodermal and paraxial mesodermal cells with a boundary directly rostral to the notochordal cells (Fig. 1A-D) Both cell layers exhibited a weaker expression of *Cdx-1* compared to the primitive streak area. The notochordal cells, as visualized in a midsagittal section, showed no *Cdx-1* expression (Fig. 1A,B).

At the head process stage (day 8.0 p.c.), *Cdx-1* expression extended laterally to the primitive streak and could be found at day 8.5 p.c. in the whole posterior part of the embryo (Fig. 2A-D). The expression anterior to the node decreased strongly (Fig. 2A,B), but could be localized at day 8.5 p.c. in the neurectoderm at the posterior hindbrain level (Fig. 2C-F).

The mesoderm exhibited a slightly weaker *Cdx-1* expression compared to the ectodermal cells in the primitive streak region. The signal was reduced further in mesodermal cells located anterior to the node, than in ectodermal cells of the developing neural tube. Nevertheless, at day 8.5 p.c., *Cdx-1* expression was detected in the developing somites (Fig. 2E,F). By day 9.5 p.c. the signal became restricted to the dorsal somitic edge of the anterior trunk somites (Fig. 2G,H). At the same embryonic stage, *Cdx-1* expression extended into the developing forelimb bud. An even distribution of *Cdx-1* transcript was detected in this structure (Fig. 2G,H). At later stages, the level of expression decreased strongly and *Cdx-1* expression could not be detected in further developed limb buds or somitic structures by in situ hybridization (data not shown).

In summary, the *Cdx-1* expression domain began in the primitive streak at day 7.5 p.c. and extended anteriorly in the neurectoderm, the somitic mesoderm and the forelimb bud during further development.

Antibody specificity demonstrated by western blot analysis

In order to examine the Cdx-1 protein distribution during embryogenesis and to characterize the endogenes Cdx-1 protein, we produced Cdx-1-specific antibodies. Based on deduced aminoacid sequences of *Cdx-1*, we selected peptides to the amino- and carboxy-terminus of the protein to raise rabbit polyclonal antisera (see Materials and Methods). To prove the specificity of the polyclonal antisera, we examined several extracts of *Cdx-1*-expressing cells and tissues by western blot analysis. Extracts were prepared from the following material: (1) 208 fibroblast cells (a rat-1 cell subclone, R. Müller personal comm.), transfected with a *Cdx-1* expression plasmid under the control of the CMV-promoter (Matthias et al., 1989); (2) embryonal carcinoma cell line F9, under undifferentiated and differentiated conditions (Strickland and Mahdavi, 1978), because *Cdx-1* expression has previously been demonstrated by northern blot analysis (data not shown) in differentiated F9 cells but not undifferentiated cells; (3) mouse embryos of day 8.5 p.c. and (4) mouse adult intestine, which both represent major *Cdx-1* expression domains.

Western blot analysis was performed with a polyclonal antisera raised to the NH_2 terminus of the Cdx-1 protein (Fig. 3) and was verified with a second polyclonal antisera raised to the COOH terminus (data not shown). The analysis revealed a band of 36×10^3 M_r specifically found in *Cdx-*

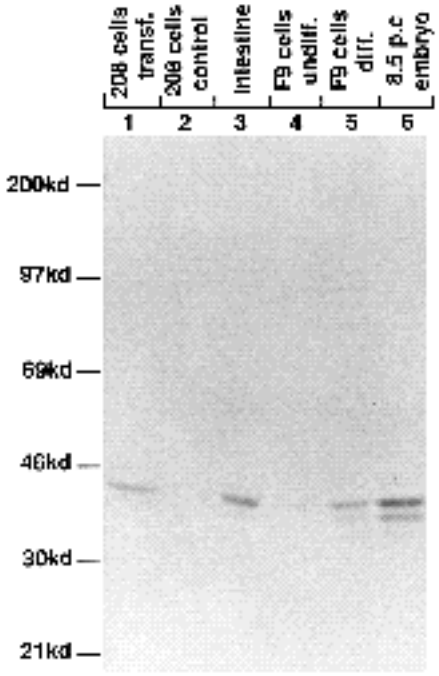


Fig. 3. Western blot analysis of the Cdx-1 protein. 30 μg cell or tissue extracts were loaded per lane. The molecular weights of the marker proteins are indicated.

1-expressing cells and tissues, while no signal was obtained in the untransfected 208 cell or undifferentiated F9 cell control extracts (Fig. 3). The lower band in lanes 5 and 6 in Fig. 3 is most likely due to degradation of the Cdx-1 protein, since freshly prepared extracts did not show this second band. The slight increase in the size of the Cdx-1 protein observed in transfected 208 cells is due to additional sequences present in the expression construct. These findings show that the Cdx-1 antisera yield one specific band in a western blot analysis, thus no cross reactivity could be observed.

Based on cDNA analysis of the *Cdx-1* gene, we predict an open reading frame of 268 amino acids for the Cdx-1 protein. The relative molecular mass is calculated to be 29×10^3. The 7×10^3 difference in the size of the predicted and observed relative molecular masses of the Cdx-1 protein could be due to the high proline content (20%) (See and Jackowski, 1989). In general, homeodomain proteins show a slower migration in SDS-PAGE gels (Erselius et al., 1990; Kessel et al., 1987).

Cdx-1 protein distribution during gastrulation

In order to investigate the distribution of the Cdx-1 protein during embryonic development, we analysed mouse embryos from day 6.5 p.c. to day 13 p.c. by immunohistochemistry performed on sections and on whole embryos with the polyclonal antisera raised against the NH_2 terminus of the Cdx-1 protein.

The earliest expression was detected at day 7.5 p.c. in the nuclei of ecto- and mesodermal cells and cells of the visceral endoderm in the area of the primitive streak (Fig. 4A-C) with the highest intensity being found in the ectodermal cells. The Cdx-1 expression domain was restricted to the immediate region of the primitive streak (Fig. 4B,C) with a slight lateral extension within the ectoderm (Fig. 4C).

At the early head process stage (day 7.75 p.c.), the Cdx-1 protein distribution extended anterior to the node in ectodermal and mesodermal cells. The anterior boundary of Cdx-1 expression in the ectoderm corresponded with the level of the preotic sulcus, a characteristic groove in the surface of the presumptive hindbrain, while the expression in the mesoderm showed a slightly more posterior boundary (Figs 4D, 6A,B). In the region of the primitive streak, a broadening of the Cdx-1 protein distribution could be observed (Fig. 4E). Consequently, the expression domain of Cdx-1 extended to the whole posterior area.

Sagittal sections showed the absence of the Cdx-1 protein in the notochordal cells (Fig. 6C,D). This observation is of particular interest, since cells of the definitive endoderm share at this stage the same basal lamina as the notochordal cells (Jurand, 1974). We found no evidence of Cdx-1 expression in cells of the definitive endoderm.

In the following paragraphs, the Cdx-1 protein distribution will be described throughout the development (day 8.25 p.c. up to day 12 p.c.) of the different expressing structures, namely the neural tube, the somites, the mesonephros and the limb bud.

Neural tube

Expression could be observed in the neural tube up to day 10.5 p.c. along its entire length (Fig. 5A). The anterior expression boundary in the neural tissue at day 8.25 p.c. to 8.75 p.c. was localized within the posterior hindbrain (Fig. 4F-I) and regressed caudally during further development to the level of the spinal cord.

Whole-mount staining of an embryo at day 8.75 p.c. revealed Cdx-1 expression in the dorsal margin of the neural folds (Fig. 4G) when the emigration of the first neural crest cells takes place (Morris-Kay and Tan, 1987). At later stages (day 9.5 p.c.) during the migration of neural crest cells in the trunk region (Bronner-Fraser and Fraser, 1991), Cdx-1 expression could be detected in cells that seem to leave the neural tube to occupy a dorsolateral position (Fig. 5B). Cross-sections showed their position in the direct vicinity of the surface ectoderm (Fig. 7B). By day 9.75 p.c., these cells represented the most anterior Cdx-1-expressing cells in the embryo within the posterior hindbrain (Fig. 5A). The major location of neural crest cells in a more ventral position exhibited no significant Cdx-1 expression (Fig. 7A,B).

Somites

All the mesodermal cells that are generated during the gastrulation process by the regressing primitive streak exhibited Cdx-1 expression, namely the segmental plate mesoderm, intermediate mesoderm and lateral plate mesoderm (Figs 4, 6).

After formation of somites, Cdx-1 expression was maintained with an equal protein distribution (Fig. 4F,G). The first three to four somites (occipital) are smaller and disaggregate by day 9.0 p.c. During this process, they seem to down-regulate the Cdx-1 expression (Fig. 4I). Thus a

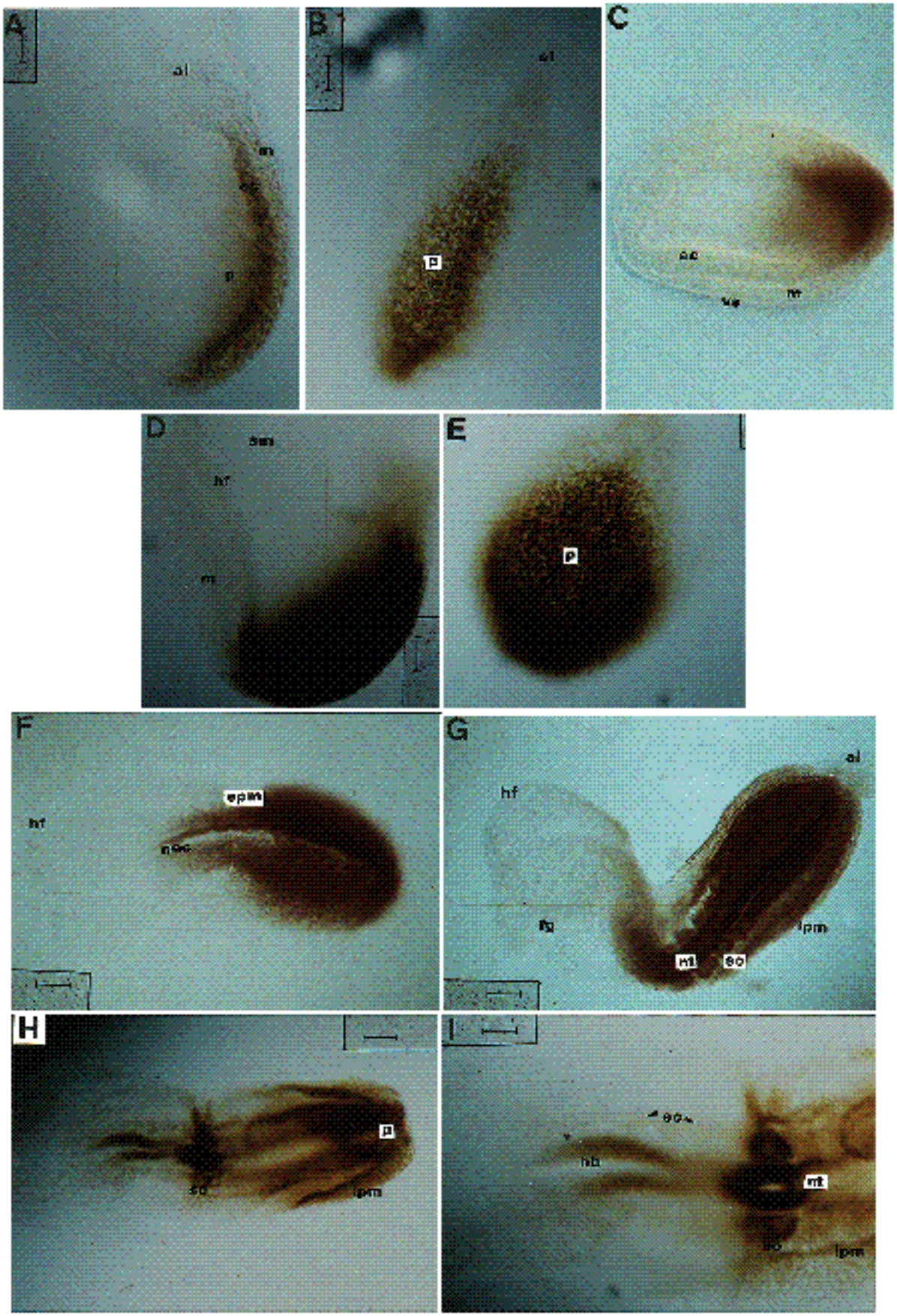

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Fig. 4. Whole-mount antibody staining of 7.5-8.75 day p.c. embryos with a Cdx-1-specific polyclonal antisera. (A-C) Different views to the same 7.5 day p.c. embryo. (A) Lateral view, the embryo is oriented, anterior to the left. The whole-mount picture is focused to a midsagittal plane of the embryo, revealing the ectodermal and mesodermal cell layer. The area of the primitive streak extends at this time point to the tip of the egg cylinder. (B) Posterior view, the tip of the egg-cylinder is down. The embryo proper is slightly visible by the weak brown contrast to the background. The structure of the allantois and the lateral sides of the embryo are out of plane, while the picture is focused on the posterior outline of the embryo. (C) Top view, anterior of the embryo is left. The whole-mount picture is focused on a transverse plane of the upper third of the embryo. (D,E) Different views of a 7.75 day p.c. embryo. The embryonic stage shows the beginning of the head-fold formation. (D) Lateral view, anterior of the embryo is left. The picture is focused on a midsagittal plane, visualizing the ectodermal cells of the headfolds and the head mesoderm. (E) Posterior view, the ventral side of the embryo is down. (A-E) Same magnification: bar, 5 μm. (F) Dorsal view on a 8.25 day p.c. embryo, anterior to the left. The picture shows the head region with the neural folds slightly visible in contrast to the background, while the more caudal part of the embryo with the neuroectoderm, the first somites, the segmental plate mesoderm and the primitive streak area are marked by the antibody reaction. The primitive streak region becomes a smaller proportion of the embryo, due to the rapid tissue increase anterior to the node. The generated segmental plate mesoderm starts to form the first somites, which will appear at the embryonic level corresponding with the rhombomere 7 in the future hindbrain. (G) Lateral view of a 8.5 day p.c. embryo, anterior to the left. The whole-mount picture shows the head region with the neural folds and the foregut region. The well-developed somites (6 to 7) are visible and the clear separation of segmental and lateral plate mesoderm becomes obvious. At this stage, the closure of the neural tube starts at the level of the fourth to the fifth somite level and the first migration of neural crest cells at the margin of the neural folds can be followed. (H,I) Ventral view of a 8.75 day p.c. embryo, anterior to the left. At this stage, the embryo starts to turn, demonstrated in this case by the ventral bending of the posterior part of the embryo. The whole mount is focused on the neural tube and somite level in the anterior part of the embryo. Due to the turning process, the plane passes through the closed neural tube and the adjacent somites in the middle and is in focus again at the posterior end of the embryo. (F-H) Same magnification, bar, 10 μm. (I) Higher magnification of the middle portion of this embryo, demonstrating the posterior hindbrain region with the disaggregating first somites. Bar, 5 μm. Abbreviations: al, allantois; am, amnion; ec, ectoderm; fg, foregut region; hb, hindbrain; hf, headfold; lpm, lateral plate mesoderm; m, mesoderm; nec, neurectoderm; nt, neural tube; p, primitive streak; so, somite; spm, segmental plate mesoderm; ve, visceral endoderm.

shift of expression to the somites at the spinal cord level could be observed by day 9.0 p.c.

By this time, the anterior somites show a concentration of Cdx-1 protein in their dorsal part (Fig. 5A-C), while the posterior somites maintained an equal protein distribution. This process continued during further development in a rostrocaudal sequence up to the level of the 16th to 17th somite: at day 9.0 p.c. the first 5 of 16 somites and at day 9.5 p.c. the first 14 of 24 somites presented this specific dorsal staining. The concentration of Cdx-1 expression to the dorsal somite area coincides with somite disaggregation. The ventromedial cell layer disperses to become sclerotome, while the dorsolateral cells remain epitheloid and form the dermomyotome (Christ and Wilting, 1992). Cross-sections revealed the strongest Cdx-1 expression in the dorsal part of the dermomyotome (Fig. 7A), while the ventral area exhibited reduced expression. Sclerotome cells that start to spread to a more medial position showed no significant signal (Fig. 7A). In the process of further somite development, the dermomyotome separates in the lateral dermatome, which gives rise to the dorsal dermis and the medial myotome (Christ et al., 1978). From the ventral edge of the myotome, cells migrate to the limb buds to produce the limb myoblasts (Chevallier et al., 1977a, 1977b; Christ et al., 1978); the remaining cells develop the myoblasts of the intercostal and flank musculature. Cdx-1 expression during this process is maintained in the dorsal somitic edge, which retains its epitheloid character. By day 12 p.c., Cdx-1 protein could be detected exclusively in this myotome structure (Fig. 7C).

This concentration of Cdx-1 expression to the dorsal somitic edge seemed to be restricted to the first 16 to 17 somites, as visualized in a day 10.5 p.c. embryo (30 somites; Fig. 5E). Posterior to this position, Cdx-1 expression decreased and increased slightly in the area of the hindlimb bud (24 to 30 somite). The *Cdx-1* gene displayed therefore a region-specific expression pattern in somites along the embryonic axis.

Mesonephros

The intermediate mesoderm situated between the paraxial mesoderm and the somatopleure or splanchnopleure gives rise to the nephrogenic cord at day 8.5 p.c., between the level of the seventh and the eleventh somite. Fig. 5C showed Cdx-1 expression in this region of a day 9.5 p.c. embryo. The pronephric tubules are already developed and the whole area exhibited weak antibody staining (Fig. 5C). At later stages (up to day 12 p.c.), the mesonephric ducts that lead to the cloaca expressed Cdx-1 (Figs 5E, 7D).

Limb buds

At day 9 p.c., the first mesodermal condensations to form the forelimb bud become visible between the 7th and the 10th somite. Cells forming this new structure derive from the lateral part of the somites (Ordahl and Le Douarin, 1992) and the somatopleure (Chevallier et al., 1977b). Both structures belong to the Cdx-1 expression domain, which now extends into the developing forelimb bud (Fig. 5A). Whole-mount immunostaining visualized an even Cdx-1 protein distribution along the anterior-posterior and the proximal-distal axis of the forelimb bud, while the dorsal part exhibited a slightly higher Cdx-1 protein concentration (Fig. 5F). Sections showed the restriction of Cdx-1 expression to the mesodermal cells; no signal could be observed in the surrounding ectodermal cells (Fig. 7A). The hindlimb bud displayed a similar Cdx-1 expression pattern along the limb axes; however, at a much lower level (Fig. 7C). At day 10.5 p.c., the forelimb bud represented the most prominent Cdx-1 expression domain, while at the same time the neural tube and somite expression had decreased (Fig. 7C).

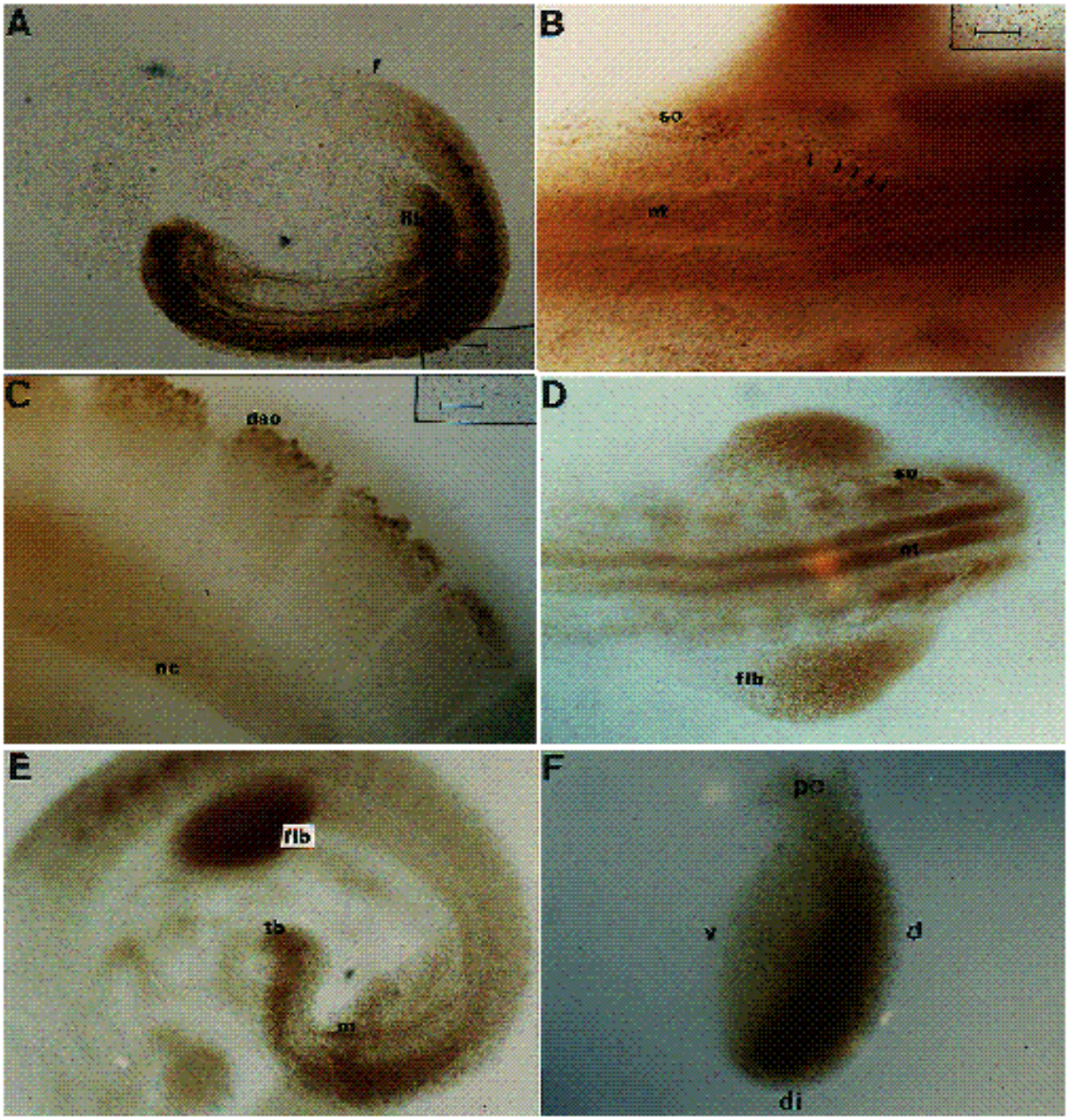

Fig. 5. Whole-mount antibody staining of 9.5-10.5 day p.c. embryos with a Cdx-1-specific antisera. (A) Lateral view of a 9.5 day p.c. embryo. The forelimb bud becomes visible at the 7th to the 10th somite level. Arrow indicates the anterior expression boundary of Cdx-1. (B) Dorsal view of a 9.75 day p.c. embryo, anterior is to the left. The picture is focused on the plane of the anterior somites and lateral cells of the neural tube, which gives the image of a transverse cut through the neural tube. The forelimb bud is visible in the back (upper part). The arrows indicate a group of cells, which occupy a dorsal position between the neural tube and the somites. (C) Lateral view around the area of the nephrogenic cord of a 9.5 day p.c. embryo, anterior to the left and dorsal to the top. This structure develops directly posterior to the forelimb bud. The somites at this embryonic level starts to disaggregate and to form the dermomyotome. (D) Dorsal view of a 9.75 day p.c. embryo, anterior to the left. The picture is focused on the forelimb bud plane. (E) Lateral view of an embryo at day 10.5 p.c., the head is missing. The picture is focused on the forelimb bud and tail bud plane, revealing the mesonephros in the posterior region. (F) Posterior view of a forelimb bud of a 10.5 day p.c. embryo. The different limb axes are indicated. Magnification: (A,E) bar 20 μm; (D,F) bar 10 μm; (B,C) bar 5 μm. Abbreviations: d, dorsal; di, distal; dso, dorsal somitic edge; flb, forelimb bud; m, mesonephros; nc, nephrogenic cord; nt, neural tube; po, proximal; so, somite; tb, tail bud; v, ventral.

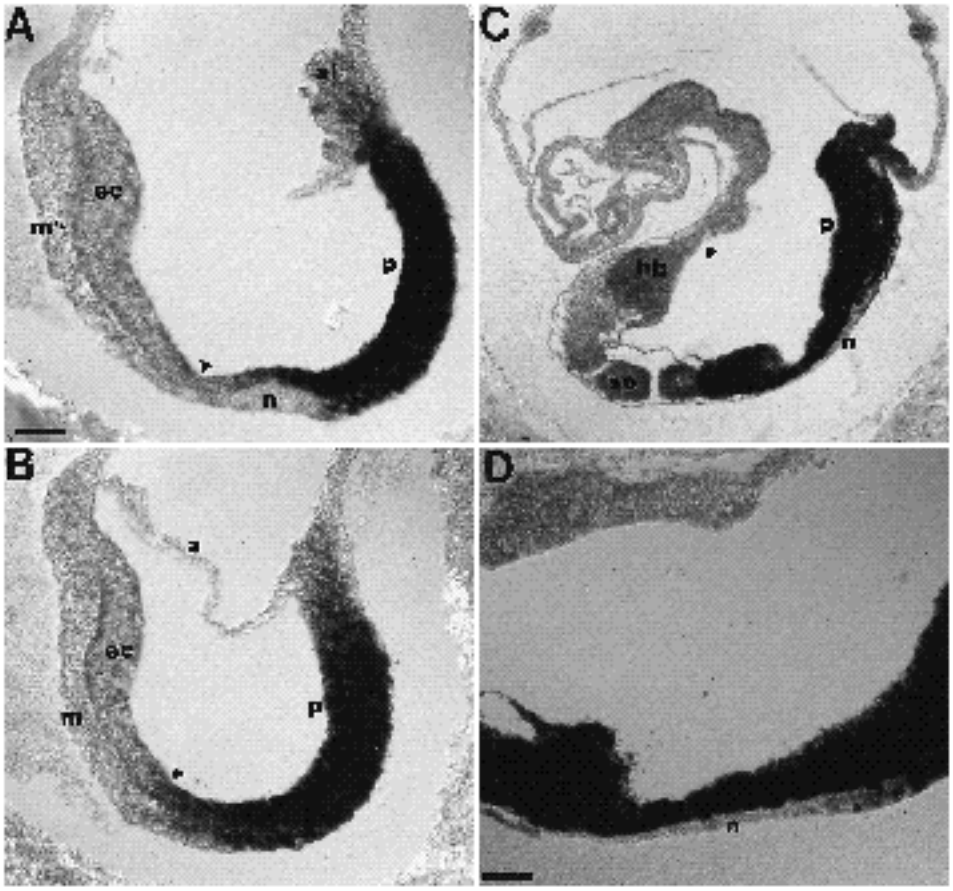

Fig. 6. Immunolocalization of Cdx-1 protein on sections of mouse embryos at day 8.0 p.c. and day 8.5 p.c. The embryo is oriented anterior to the left. (A) Midsagittal section of a 8.0 day p.c. embryo. Arrow indicates the preotic sulcus. (B) Parasagittal section of the same embryo as in A. (C) Sagittal section of an embryo at day 8.5 p.c. The embryonic plane of this section crosses, at the level of the segmental plate mesoderm, the midline of the embryo, indicated by the visible notochordal cells in this area. (D) Higher magnification of a section as shown in C, in the area of the segmental plate mesoderm, anterior to the node. Magnification: (A-C) bar 10 μm; (D) bar 5 μm. Abbreviations: a, amnion; al, allantois; ec, ectoderm; hb, hindbrain; m, mesoderm; n, head process/notochord; p, primitve streak; so, somite.

DISCUSSION

In higher vertebrates, the process of gastrulation is marked by the formation of the primitive streak, which plays a key role in the establisment of the embryonic axis. In an attempt to get a better insight, we have analysed the expression pattern of the *Cdx-1* gene during early development by following its RNA and protein distribution. The *Cdx-1* expression began at day 7.5 p.c. (head process stage) and was restricted to the area of the primitive streak. By the time Cdx-1-expressing ectodermal and mesodermal cells occupy a position anterior to the node and begin to develop into neurectoderm and somitic mesoderm, the *Cdx-1* expression was reduced. The anterior expression boundary in the neural tissue corresponded with the preotic sulcus, and regressed caudad to the spinal cord level during development (Fig. 8). The somite-specific expression could be followed until day 12 p.c. in the dorsal somitic edge. With the development of the limb buds *Cdx-1* expression extended into these structures. No expression could be detected in the developing notochord.

Up to day 9.5 p.c., RNA and protein analyses yield coincident pattern; however, at later stages, due to the lower sensitivity of the in situ hybridization, we failed to reveal *Cdx-1* expression. Northern analysis (Duprey et al., 1988), performed on embryonic tissues from day 10 to 17 p.c., shows *Cdx-1* transcripts at day 10 p.c. The signal decreases to undetectable levels between day 11 and 14 p.c. These data were confirmed with the antibody staining, which showed a strong decrease of Cdx-1 expression after day 10.5 p.c.

Comparison of *Cdx-1* to *CHox-cad*

To date, a gastrulation-specific expression of a caudal-type homeobox gene is described in detail only for the *CHox-cad* gene in chicken (Frumkin et al., 1991). The activity of this gene commences in the elongating primitive streak at the onset of gastrulation. Later in development, the *CHox-cad* expression is restricted to the epiblast cells of the primitive streak and the definitive endodermal cells, suggesting a *CHox-cad* involvement in the formation of the endodermal germ layer.

In contrast, we could show that the mouse *Cdx-1* gene is expressed in ectodermal and mesodermal-derived structures and not in cells that give rise to the definitive endoderm. Cell mapping experiments in mouse demonstrated that the cells of the definitive endoderm derive from the epiblast (Beddington, 1983) and that the majority of these cells migrate through the primitive streak from day 6.5 to 7.5 p.c. (Lawson et al., 1991). During this time, *Cdx-1* expression was undetectable. The fundamental differences in the early expression patterns of the mouse *Cdx-1* and the chicken *Chox-cad* gene indicate that they represent different members of the vertebrate caudal-type homeobox class. This is further support by the low amino acid sequence homology of 73.4 % (conserved exchanges included). In the mouse genome, beside *Cdx-1*, another member of this class has been isolated, the *Cdx-2* gene (James and Kazen-

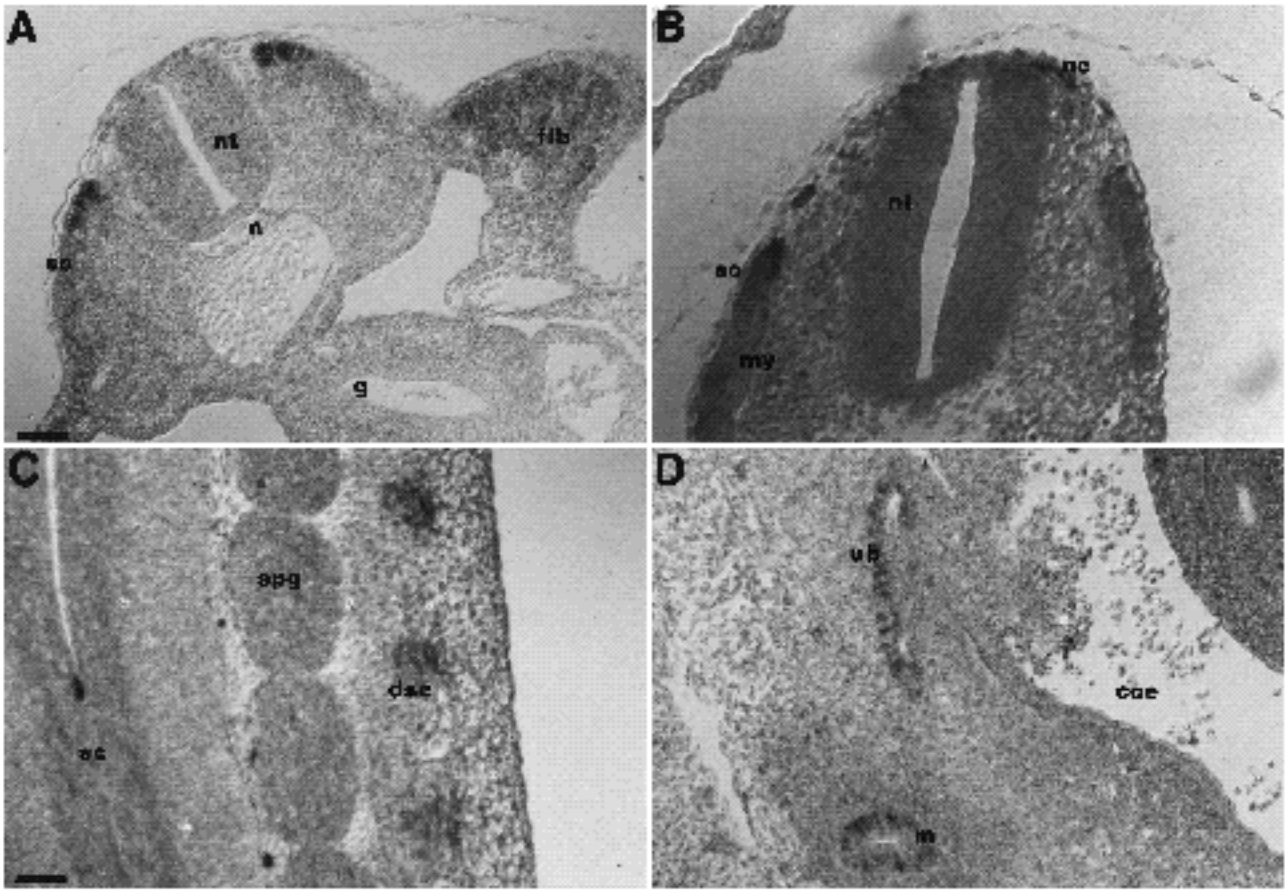

Fig. 7. Immunolocalization of Cdx-1 protein on sections at various embryonic stages. (A) Transverse section of a 9.5 day p.c. embryo at the level of the forelimb bud. (B) Transverse section of a 10.5 day p.c. embryo anterior to the forelimb bud. (C) Transverse section of a 12 day p.c. embryo, presenting the spinal cord and spinal ganglia. (D) Sagittal section of a 12 day p.c. embryo in the urogenital area. Magnification: (A,B) bar 10 μm; (C,D) bar 5 μm. Abbreviations: coe, coelom; dse, dorsal somitic edge; flb, forelimb bud; g, gut; m, mesonephros; my, myotome; n, notochord; nc, neural crest; nt, neural tube; sc, spinal cord; so, somite; spg, spinal ganglia; ub, ureteric bud.

wadel, 1991). However, the expression pattern of *Cdx-2* has only been reported in adult tissues, where it is found in the intestine. The limited *Cdx-2* sequence data available thus far do not allow comparative analysis to the *CHox-cad* gene.

Since *Cdx-1* and *CHox-cad* show mostly non-overlapping expression pattern, different functions of caudal-type homeobox genes in vertebrates are likely.

Cdx-1 expression and the primitive streak

Cdx-1 expression during gastrulation commenced at day 7.5 p.c. within the primitive streak. This time point coincides with the start of regression or head process formation. Based on cell lineage studies in chicken (Stern and Canning, 1990; Stern et al., 1988) and mouse (Beddington, 1983; Lawson et al., 1991; Tam, 1989), it is proposed that, prior to streak regression (day 7.5 p.c. in mice), the embryonic ectoderm is already laid down to form the forebrain, midbrain, hindbrain and spinal cord in a correct craniocaudal order along the A-P axis. Nevertheless, it has been concluded that most embryonic ectoderm is not committed to a specific fate at this time, since grafting experiments in mice of prospective forebrain/midbrain neurectoderm showed that the transplants adopt the cellular fates of their new sites (Beddington, 1982). With the start of neurulation, visible by the appearance of the node and the notochordal cells, the rostrocaudal positional assignment begins. The onset of *Cdx-1* expression at this time point, provides us with a marker of differential gene activity established as a consequence of A-P positional specification.

Cdx-1 expression at primitive streak stages showed the highest intensity in ectoderm with a slightly anterior and lateral extension compared to mesoderm, suggesting that the gene was initially activated in the ectodermal cells. However, during primitive streak regression, the expression extended to the whole embryo caudal to the posterior hindbrain (with the exception of the notochordal cells; Fig. 8). Therefore, the relation of the onset of gene activity with the spatial domain of expression in the developing tissues, indicates an involvement of *Cdx-1* in mediating positional information to the ingressing cells of the primitive streak beginning at day 7.5 p.c. The only cells in which *Cdx-1* expression is absent during this process are the notochordal cells, which are generated by the ingression of ectodermal cells through

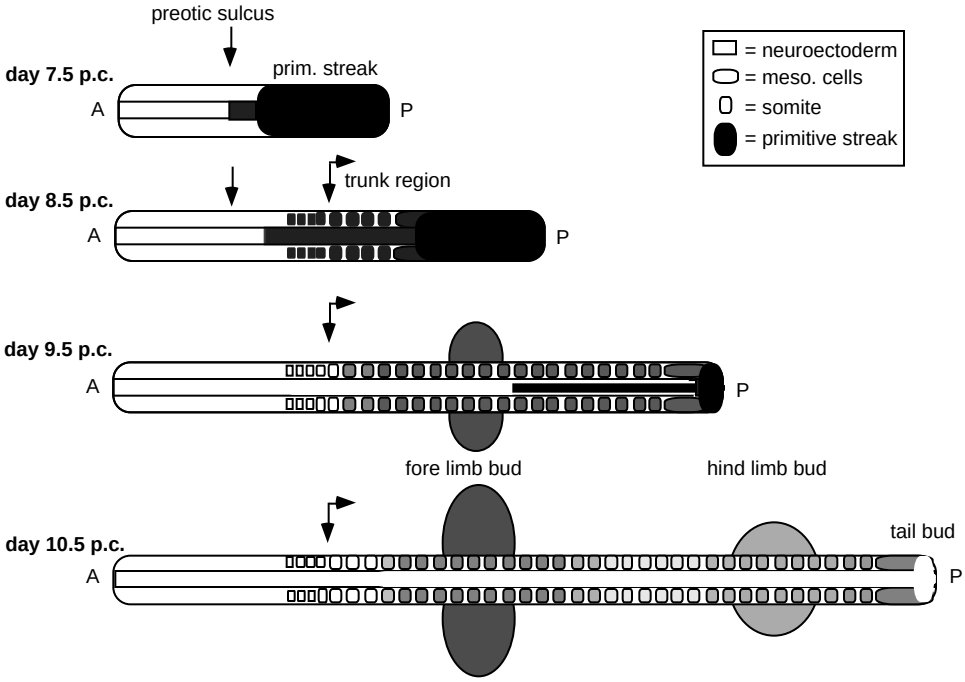

Fig. 8. Schematic summary of the *Cdx-1* expression pattern during early mouse development. *Cdx-1* expression is indicated by grey to black pattern reflecting lower or higher protein levels. The scheme presents a dorsal view of the embryonic axis.

the node (Selleck and Stern, 1991, 1992). These cells are associated with neural induction of the overlying ectoderm (Gurdon, 1987). The activity of the *T-gene* (Herrmann et al., 1990; Wilkinson et al., 1990) in mice has been correlated with the development of notochordal cells, because the homozygote *T-gene* mutant *Brachyury* (for review see Lyon and Searle, 1989; Dobrovolskaia-Zavadskaia, 1927) lacks a notochord. While *Cdx-1* and *T* exhibit overlapping expression domains in the primitive streak, they show complementary expression patterns in the tissues anterior to the node. Therefore, both genes provide us with useful markers which could help to understand the differentiating pathways of notochordal and somitic mesodermal cells.

Cdx-1 expression along the embryonic axis

Consistent with the start of neurulation, *Cdx-1* was expressed along the entire embryonic axis posterior to the middle of the prospective hindbrain. While the embryo continues to develop in an rostral-to-caudal direction, the anterior expression boundary retreated to the spinal cord level. This process occurs during morphological differences in the hindbrain (rhombomeres develop by day 8.5 to 9.0 p.c.) become visible (Fraser et al., 1990; Lumsden and Keynes, 1989). The expression pattern of Hox genes coincides with the compartmentalization of the hindbrain beginning in the 8 p.c. day embryo (Murphy and Hill, 1991; Wilkinson et al., 1989). Therefore, the *Cdx-1* expression pattern indicates a possible relation to the expression of clustered Hox genes. The beginning of *Cdx-1* expression on day 7.5 p.c. coincides with the appearance of the first Hox gene transcripts, namely of *Hox-1.6* (Hunt et al., 1991; Sundin et al., 1990) and *Hox-2.9* (Frohman et al., 1990; Guthrie and Lumsden, 1991). While *Cdx-1* expression was detectable in the primitive streak sequentially, more and more Hox genes are being activated. Anterior of the node where the final establishment of axial identities coincides with the fixation of Hox expression domains, *Cdx-1* expression was turned off. This observation suggests that *Cdx-1* might play a role in the determination of the embryonic axis in its initial phase, because it disappears when axial positions are specified. This interpretation is further supported by the *Cdx-1* expression pattern within the developing limb buds. Again *Cdx-1* activity was only detectable at the beginning of limb outgrowth. Differing from the 5′ located members of the *Hox-1* (Yokouchi et al., 1991; Haack, in preparation) and *Hox-4* cluster (Izpisuá-Belmonte et al., 1991, 1992), which show a temporal and spatial restricted expression pattern in the limb, *Cdx-1* exhibited an uniform expression pattern, suggesting a function in axis formation rather than specification.

In summary, the temporal and spatial expression pattern of *Cdx-1* during gastrulation suggests an involvement in determination of the embryonic axes. The coincidence of expression with 3′ located members of the Hox clusters is striking, while their major feature of establishing a defined anterior expression boundary, is not realized by *Cdx-1*. The fundamental differences in the expression pattern of mouse *Cdx-1* and chicken *CHox-cad* demonstrate the different functions of caudal type homeobox genes in vertebrate development.

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