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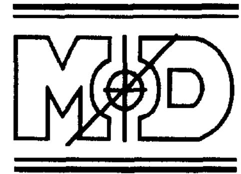

The restricted expression pattern of the POU factor Oct-6 during early development of the mouse nervous system

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Abstract

Oct-6 is a POU transcription factor that is thought to play a role in the differentiation of cells of neuroectodermal origin. To investigate whether the Oct-6 protein could play a role in the establishment of neuroectoderm in vivo we studied the expression of the Oct-6 protein during early mouse development. Expression is first observed in the primitive ectoderm of the egg cylinder stage embryo. In gastrulating embryos, Oct-6 protein is found in the extra-embryonic ectoderm of the chorion and the anterior ectoderm of the embryo proper. As development proceeds, Oct-6 expression becomes more restricted to the anterior medial part of the embryo until Oct-6 positive cells are observed only in the neural groove of the headfold stage embryo. In the late headfold stage embryo, Oct-6 expression is detected in the neuroepithelium of the entire brain and later is restricted to a more ventral and anterior position. As the anterior neuropore closes, Oct-6 protein is detected in a segment-like pattern in the mid- and forebrain. Thus, the expression pattern of the Oct-6 gene agrees with a role for the Oct-6 protein in the establishment and regional specification of the neuroectoderm in vivo. The two waves of widespread induction of the Oct-6 gene, one in the primitive ectoderm and another in the primitive brain, both followed by a progressive restriction in the expression patterns suggest a mechanism for the regulation of the gene.

Keywords: Oct-6; POU domain; Neuroectoderm; Brain development; Prosomere

1. Introduction

Genes involved in neurogenesis have been the subject of intense study in many different organisms. Many of these genes are members of large families and encode DNA binding proteins that could act as transcriptional regulators. These different gene families are defined by their evolutionary conserved DNA binding domain. One of these families, the POU family, consists of a growing number of tissue specific DNA binding proteins that share the conserved POU DNA binding domain (Herr et al., 1988; Schöler, 1991; Wegner et al., 1993). The POU domain is a 150–160 amino acid region containing a 60 amino acid POU-homeodomain (POU_{HD}), highly homologous with the classical homeobox, and a 75–82 amino acid POU-specific domain (POU_{SP}). The amino terminal POU_{SP} domain is separated by a short linker sequence (15–27 amino acids) from the carboxyl terminal POU_{HD} domain. Based on homologies within the POU domain these genes have been grouped in six classes (classes I–VI; Wegner et al., 1993). More than 13 mammalian POU family members have been identified and POU genes have also been cloned from the genomes of *Drosophila* (Johnson and Hirsh, 1990) *C. Elegans* (Burglin et al., 1989), *Bombyx mori* (Fukuta et al., 1993), zebrafish (Johansen et al., 1993; Hauptmann and Gerster, 1995), chicken (Petryniak et al., 1990) and frogs (Agarwal and Sato, 1991). During development most POU genes show a tissue specific expression pattern, suggesting that they are involved in cell type determination or regional specification. They will function during cell differentiation, regulating the tissue specific expression of downstream genes through interaction with octamer and octamer-like DNA elements (Rosenfeld, 1991; Wegner et al., 1993).

We have been studying the Oct-6 gene, which together with the POU genes Brn-1, Brn-2 and Brn-4, form the mammalian class III POU family. We identified the

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mouse POU factor Oct-6 as an octamer binding protein that is differentially expressed during neuroectodermal differentiation of P19 embryonal carcinoma cells (Meijer et al., 1990). The rapid induction of this gene in differentiating cells followed by a progressive downregulation and the reappearance of expression in fully differentiated cultures suggest that Oct-6 is involved in different aspects of neuronal differentiation. Furthermore, Oct-6 is expressed in non-differentiated embryonic stem cells that are derived from the inner cell mass (ICM) of blastocyst stage embryos (Meijer et al., 1990; Suzuki et al., 1990). The rat homolog, called SCIP or Tst-1, cloned from rat testis (He et al., 1989), was shown to be a cAMP (cyclic adenosine monophosphate) inducible gene in cultured neonatal Schwann cells isolated from the sciatic nerve (Monuki et al., 1989).

Several studies have described the expression of the Oct-6 gene in the developing and adult central nervous system using RNA in situ histochemistry. During mid and late gestation periods of mouse embryonal development, Oct-6 RNA is found in the subventricular zones in the brain. Expression is most abundant in prospective cerebral cortex, olfactory bulb, striatum, thalamus and inferior colliculus (Suzuki et al., 1990). In rat a similar embryonic expression pattern was observed (He et al., 1989). Expression in the adult rat cerebral cortex is very prominent in layer 5 pyramidal neurons and is detected as these cells originate in the intermediate zone during embryonal development (Frantz et al., 1994). These RNA in situ studies suggest that the Oct-6 gene product could be involved in determination of neural identity. In addition, the immediate induction of Oct-6 gene expression in P19 cell aggregates upon retinoic acid (RA) addition, suggests that Oct-6 could be involved in the differentiation of neuroectodermal derivatives from a multipotent embryonic stem cell. However, very little is known about the expression of the Oct-6 gene during early mouse development.

To investigate whether the expression pattern of Oct-6 protein (rather than RNA) agrees with a role of the Oct-6 gene in the establishment of the neuroectoderm, we performed immunohistochemical staining of early mouse embryos, using a polyclonal antiserum directed against the Oct-6 protein.

2. Results

2.1. Oct-6 polyclonal antiserum

A polyclonal antiserum was raised in rabbits against bacterially produced, denatured Oct-6. First, the reactivity of the antiserum with mouse Oct-6 protein was tested in a band shift assay (Fig. 1a). A nuclear cell extract of the Mes68 cell line expressing the POU proteins Oct-1 and Oct-6 was incubated with a radiolabeled double stranded oligonucleotide containing the octamer ATTTGCAT POU protein consensus binding site. After protein-DNA complexes had formed the Oct-6 antibody was added to the binding reaction. The mobility of the Oct-1 and Oct-6 protein complexes was found to be unaltered when preimmune serum was added (Fig. 1a, compare lanes 1 and 2). However, addition of Oct-6 antibody gives rise to a complete retardation of the Oct-6 protein complex in the top of the gel, whereas the Oct-1 complex was unaffected (Fig. 1a, lane 3). This experiment shows that the Oct-6 antiserum is able to recognize mouse Oct-6 protein. Furthermore the antiserum is highly specific since it does not cross-react with Oct-1 protein despite the presence of a large excess of Oct-6 antibodies.

To further ensure the specificity of the antiserum we performed an affinity purification. Because the antiserum was raised against the full length Oct-6 protein we were concerned with the possibility of cross-reaction with other POU proteins through conserved epitopes within the POU domain. In addition to affinity purification we competed the purified Oct-6 antibodies with a cellular lysate of bacteria overexpressing the Oct-6 POU domain (Oct-6/POU) during the antibody incubation period in the immunohistochemistry experiments. To determine the efficiency of this method, a similar competition was performed in a western blot assay (see Fig. 1b). When whole cell extracts of transiently transfected COS-1 cells overexpressing either the Oct-6 POU domain (lanes 2 and 4) or the full length Oct-6 protein (lanes 1 and 3) were stained with the affinity purified Oct-6 antiserum, both Oct-6 and the Oct-6 POU domain were detected when a lysate of non-induced bacteria was used as a competitor (lanes 1 and 2). However, the Oct-6/POU competitor completely eliminated detection of the POU domain,

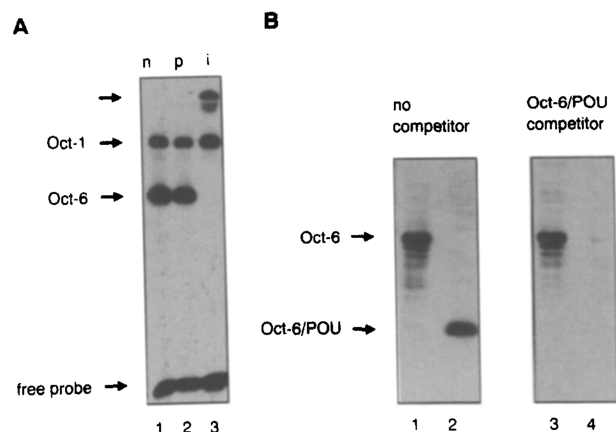

Fig. 1. Oct-6 antibodies specifically recognize Oct-6 protein. (A) A band shift experiment of Mes68 nuclear cell extract and a radiolabeled Octamer oligo. Lane 1, no serum was added; lane 2, 1 μl preimmune serum; lane 3, 1 μl Oct-6 antiserum. The arrow indicates the retarded Oct-6 protein complex. (B) A western experiment detecting either Oct-6 protein (lanes 1 and 3) or Oct-6/POU (lanes 2 and 4) under competitive conditions of a non-expressing (lanes 1–2) or Oct-6/POU (lanes 3–4) expressing bacterial lysate.

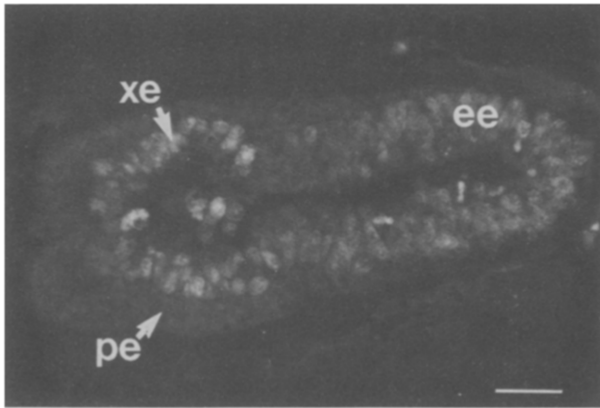

Fig. 2. Oct-6 expression in a prestreak embryo. The figure shows an oblique sagittal section of a 5.5 dpc embryo. Oct-6 is expressed in the embryonal and extra-embryonal ectoderm. The bar represents 25 μm. Abbreviations: ee, embryonal ectoderm; pe, proximal endoderm; xe, extra-embryonal ectoderm.

leaving detection of the full length Oct-6 protein unaffected (lanes 3 and 4). This experiment demonstrates that addition of the POU competitor efficiently blocks the epitopes in the conserved POU domain while other Oct-6 specific epitopes are still recognized by the Oct-6 antiserum. Since the Oct-6 POU DNA binding domain is the only region with significant homology to its most closely related POU family members, this method eliminates any cross reactivity with other POU proteins. That this is also the case in the immunohistochemistry experiments comes from a number of observations not shown here. First, the closely related class III POU proteins Brn-1 and Brn-4 are highly expressed in the developing kidney and otic vesicle, respectively (He et al., 1989; Le Moine and Young, 1992). No signal is detected with our Oct-6 antibody in these structures. Second, the Brn-3 genes are highly expressed in the sensory ganglia (Ninkina et al., 1993). No signal is detected with our antibody. Third, the Oct-3/4 gene is highly expressed in the inner cell mass and trophectoderm (Schöler et al., 1990). Again no signal is detected with our antibody in these structures. These and numerous other observations demonstrate that the antibody specifically recognizes the Oct-6 protein and none of the other known POU proteins.

2.2. Oct-6 expression in presomite stage embryos

The Oct-6 gene is expressed in inner cell mass (ICM) derived ES cells (Schöler et al., 1989; Meijer et al., 1990). To test whether the Oct-6 protein is present in the ICM of pre-implantation blastocyst embryos we performed immunohistochemistry on whole mount blastocyst embryos. Using our polyclonal Oct-6 antiserum, no Oct-6 protein could be detected in these embryos (data not shown). This was not unexpected since a previous paper reported the failure to detect Oct-6 mRNA in these preimplantation embryos (Suzuki et al., 1990). However we could detect the Oct-6 protein in the nuclei of embryonic and extra-embryonic ectodermal cells of a 5.5 dpc egg cylinder stage embryo (Fig. 2). Thus a major period of onset of expression must occur sometime after or during implantation and subsequent expansion of the epiblast into a cylindrical structure.

In late streak embryos, Oct-6 protein is found in the anterior half of the embryo. No expression is found in the posterior and lateral-posterior parts of the embryo (Fig. 3a). Transverse sections of embryos of similar developmental stage revealed that Oct-6 protein is expressed in the cells of the ectodermal layer (data not shown). A sagittal section shows that the posterior boundary of expression extends to the distal tip of the midstreak embryo (Fig. 3e). This position coincides with the position of the node. In more advanced embryos the lateral expression boundaries progressively restrict to a more anterior position (Fig. 3b,c). Furthermore, a stripe of non-expressing cells becomes visible at the midline of the embryo anterior of the node (Fig. 3f). This becomes even more evident in the late headfold stage embryo shown in Fig. 3c as the lateral boundary of expression has regressed to the parallel neural ridges in the headfold. Thus, during gastrulation and subsequent neurulation Oct-6 protein expression is progressively restricted to the walls of the neural groove after initial widespread expression in the primitive ectoderm.

Besides expression in the embryonal ectoderm, Oct-6 protein is also found in the extra-embryonal ectoderm. As shown in Fig. 3a–f, Oct-6 is expressed in the ectodermal component of the chorion membrane. This extra-embryonal tissue derives from both the ecto- and mesoderm and fuses with the allantois and ectoplacental cone to form the placenta.

2.3. Oct-6 expression in 5–17 somite-pair stage embryos

Formation of the head folds is initiated by invagination of the foregut and is characterized by exceptionally rapid growth of the neuroepithelial cells (see Snell and Stevens, 1966). In 5 somite embryos, Oct-6 protein is highly expressed in the head folds (Fig. 4a). Expression extends to the anterior extremity of the neuroectoderm (Fig. 4b) and is evenly distributed along the entire medial to lateral (M–L) axis of the headfolds (Fig. 4a). Proceeding in development, Oct-6 protein expression regresses in the hindbrain region in a ventral and anterior direction (Fig. 4c,d). The posterior boundary of expression is indicated with arrowheads in Fig. 4c,d and is located rostral of rhombomere 6. In the lateral extremities of the head folds the lateral and anterior boundaries of expression are regressing, leading to a more ventral position of Oct-6 expressing cells in the neuroepithelium (Fig. 4c). When the rostral neuropore has closed, Oct-6 expression becomes restricted to the developing fore- and midbrain.

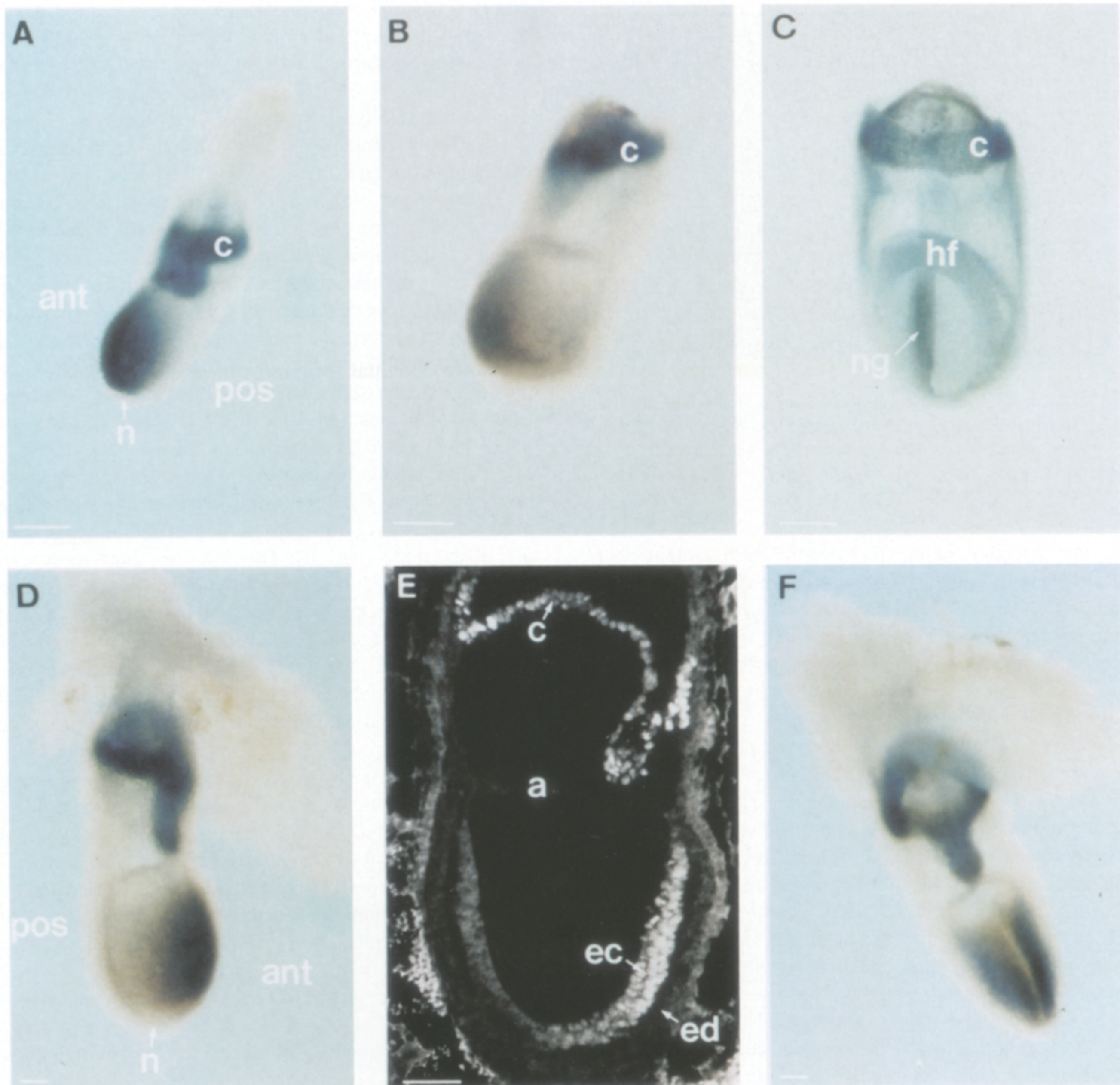

Fig. 3. Progressive restriction of Oct-6 in the anterior ectoderm during gastrulation. (A–C) Lateral views of whole mount embryos stained for Oct-6 expression. Anterior is to the left. Expression restricts from a rather widespread distribution in the ectoderm to the neurepithelial cells of the neural groove in a head fold embryo. (A) Late streak, (B) early bud neural plate and (C) advanced head fold stage embryos. The bar represents 400 μm. (D,F) A lateral and anterior view, respectively, of a no bud neural plate stage embryo. (E) A sagittal section of a different embryo of similar stage. In (D) and (E) anterior is to the right. Expression is confined to the embryonal ectoderm and extra-embryonal chorion membrane and is absent from the midsagittal streak. The bar represents 200 μm. Abbreviations: a, amnion; ant, anterior; c, chorion; ec, ectoderm; ed, endoderm; hf, head fold; ng, neural groove; n, node; pos, posterior.

2.4. Oct-6 expression in 9 to 10.5 dpc developing forebrain

At 9.5 dpc Oct-6 protein expression has stabilized in two domains within the mid- and forebrain. A posterior domain encompassing the ventral aspect of the rostral mesencephalon and caudal diencephalon. A second, more anterior expression domain is seen at the ventral midline of the secondary prosencephalon which extends at later stages into the telencephalon. (Fig. 5a). A frontal section of a 9.5 dpc embryo shows that in the posterior expression domain Oct-6 is detected in the basal plate of mesencephalon and diencephalon (Fig. 5b). Furthermore a number of Oct-6 positive neurons can be seen at a dorsal position in the mid and forebrain (see Fig. 5a). Sagittal and transverse sections show (Fig. 5c,d) that the anterior

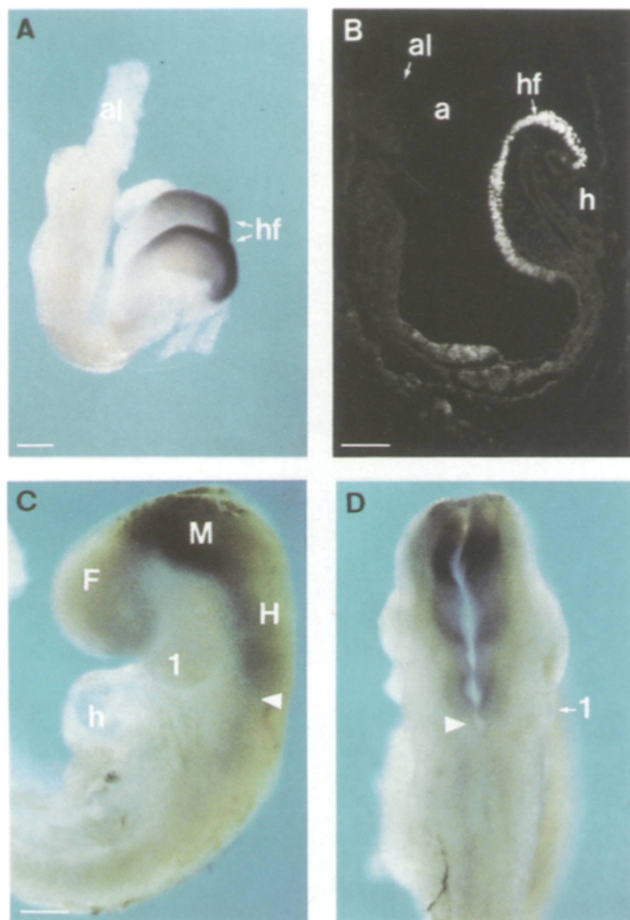

Fig. 4. Oct-6 expression in the future brain region in 5 and 17 somites stage embryos. (A,B) Oct-6 expression in a 5 somite embryo. (A) A lateral view of a whole mount stained embryo. Anterior is to the right. (B) A sagittal section. (C,D) A lateral and dorsal view, respectively, of a 17 somite embryo. The arrowhead points at the posterior expression boundary. Bar in (A–D) represents 500 μm. Abbreviations: a, amnion; al, allantois; F, forebrain; H, hindbrain; h, heart; hf, head fold; M, midbrain; 1, first branchial arch.

expression domain of Oct-6 is restricted rostral of the infundibular recess and caudal of the optic chiasm. Furthermore the oblique transverse section shown in Fig. 5d indicates that Oct-6 expression is limited by a sharp lateral boundary. The infundibular recess and Rathkes pouch do not express Oct-6.

Recently, the expression patterns of a large number of genes have been described in the developing forebrain. The boundaries of mRNA expression would reflect in part a segmental organization of the forebrain as was proposed by Puelles and Rubenstein (1993) and Figdor and Stern (1993). In these models, the forebrain is segmentally organized by six transverse prosomeres or four neuromeres which are subdivided by four longitudinal segments that follow the body axis. To study whether Oct-6 expression is confined to such segments or prosomeres, we performed whole mount RNA in situ hybridization experiments using an Oct-6 and Sonic hedgehog (Shh) probe. We used Shh as its expression has been described in detail (Echelard et al., 1993) , and furthermore provides a convenient marker for the border between prosomere 2 and 3 (at the zona limitans intrathalamica (ZLI) = border between domain D1 and D2 in Figdor and Stern) and the ventral midline of the rostral diencephalon (also called the secondary prosencephalon). Whole mount RNA in situ hybridization of 9.5 dpc embryos using Shh, Oct-6 or Shh + Oct-6 anti-sense RNA probes indicates that the rostral boundary of the caudal Oct-6 expression domain coincides with the lateral extensions of Shh expression that mark the boundary between prosomere 2 and 3 (Fig. 6a,c,e). Sense RNA probes did not detect any signal (data not shown). Sagittal sections of these embryos show that the rostral Oct-6 expression domain falls within the Shh expression domain at the ventral midline of the rostral diencephalon (Fig. 6b,d). These results are schematically summarized in Fig. 6f. Thus Oct-6 expression in the forebrain seems to be restricted to the basal segments of prosomere 1, 2 and 6 according to the scheme of Puelles and Rubenstein. The expression of Oct-6 in the ventral midline of the rostral diencephalon is maintained at later stages of development but extends rostrally into the telencephalon.

3. Discussion

Based on in vitro cell culture systems and mRNA expression studies in mid to late gestation embryos, it has been suggested that the POU factor Oct-6 plays a role in various aspects of neuronal differentiation. The rapid induction of Oct-6 mRNA in RA-treated P19 cell aggregates suggests that the gene product could also be involved in the establishment of the neuroectoderm in vivo. In this report we have described the expression pattern of the Oct-6 protein during early mouse development, from the pregastrulation stage until midgestation, using highly specific affinity purified antiserum in immunohistochemistry experiments.

3.1. Oct-6 and ectoderm differentiation

During gastrulation and subsequent neurulation, we found that the expression of Oct-6 protein progressively restricts from a rather ubiquitous expression pattern in the primitive ectoderm to the neuroectodermal cells in the neural groove of a head fold stage embryo. It appears that Oct-6 expression stabilizes in cells that are fated to develop into neuroectoderm whereas expression is extinguished in other ectodermal derivatives. This suggests that Oct-6 plays a role in the progression of neuroectodermal differentiation.

The progressive restriction of Oct-6 protein expression to the anterior part of the embryo is reminiscent of the ectodermal expression of the Otx2 gene (Ang et al., 1994). Otx1 and Otx2 are the mammalian homologs of

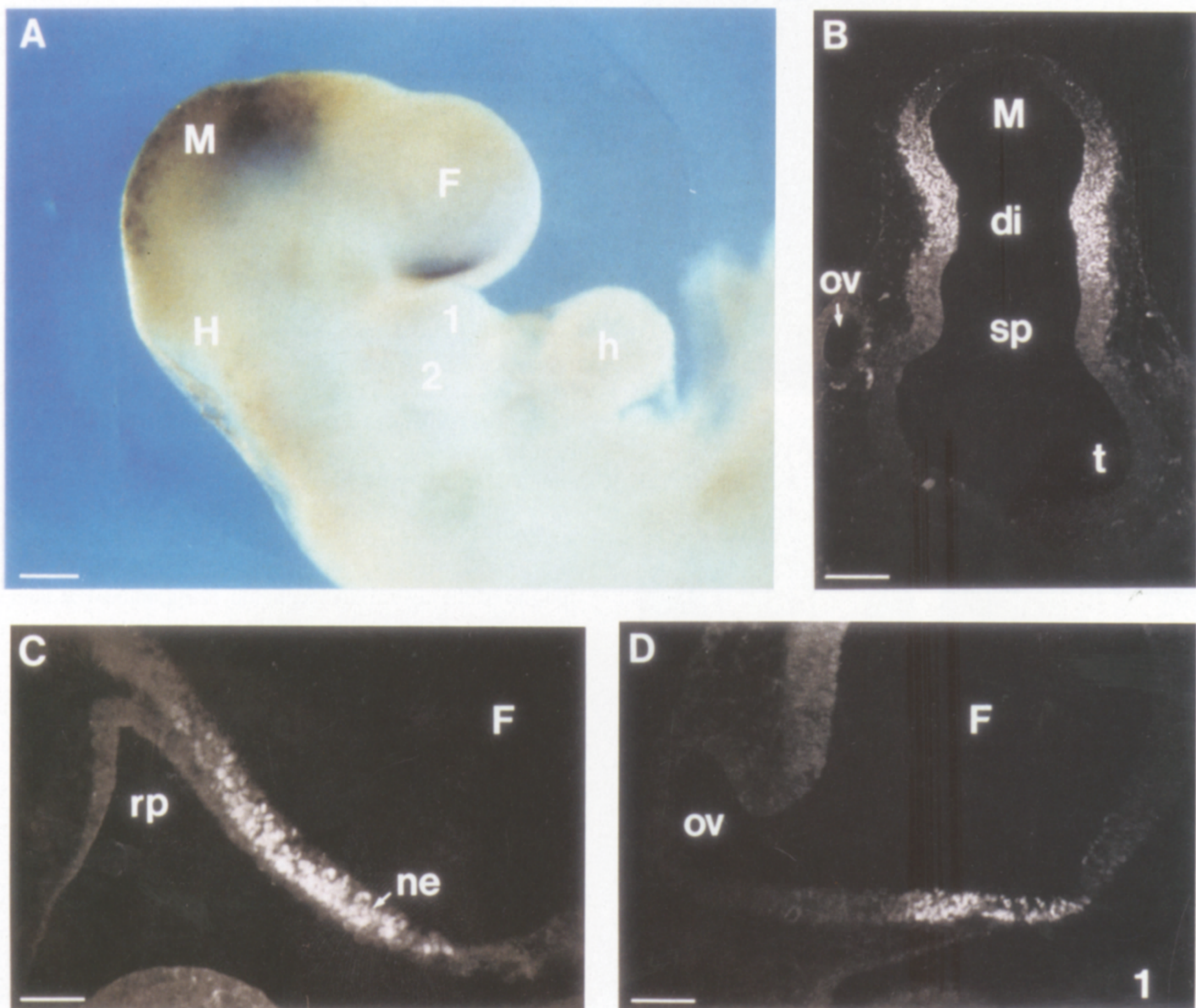

Fig. 5. Oct-6 expression in the developing forebrain of a 9.5 days pc embryo. (A) A ventrolateral view of a 9.5 days pc embryo. In (B) a frontal section is shown through the fore- and midbrain. In (C) sagittal sections show expression in the neuroepithelium of the ventral floor of the secondary prosencephalon anterior of the infundibular recess. A transverse section shows that the expression has clear lateral boundaries of expression (D). Bar in (A) and (B) represents 500 μm and in (C) and (D) 150 μm. Abbreviations: di, diencephalon; F, forebrain; H, hindbrain; h, heart; M, midbrain; ne, neurectoderm; ov, optic vesicle; rp, Rathke's pouch; sp, secondary prosencephalon; t, telencephalon; 1, first branchial arch; 2, second branchial arch.

the *Drosophila* homeobox gene orthodenticle which is involved in the patterning of anterior head structures. Explant-recombination experiments performed by Ang et al. indicate that the progressive restriction of Otx2 expression to the anterior part of the mouse embryo is brought about by a negative signal emanating from the posterior mesendoderm (Ang et al., 1994). This parallel in expression pattern suggests that Oct-6 is similarly regulated and possibly responds to the same signal that downregulates Otx2.

The restriction of Oct-6 protein expression during gastrulation and neurulation is followed by a second round of induction of the gene in the anterior neuroectoderm. As the embryo develops from a late headfold stage to early somite stage, Oct-6 expression is detected in the primitive brain. This induction of high levels of Oct-6 expression coincides with the rapid proliferation of the anterior headfolds (Stevens and Snell, 1966). Again this widespread expression becomes restricted as the brain further develops and adopts a segment-like pattern in the mid- and forebrain at the time the anterior neuropore closes. Thus, it appears that the early embryonic expression pattern of Oct-6 is the result of two separate waves of induction, both followed by a progressive restriction through negative regulation of the gene. The molecules that execute these functions and the mechanisms by which they act are completely unknown. The identification of the *cis*-acting DNA elements in the Oct-6 locus that regulate the devel-

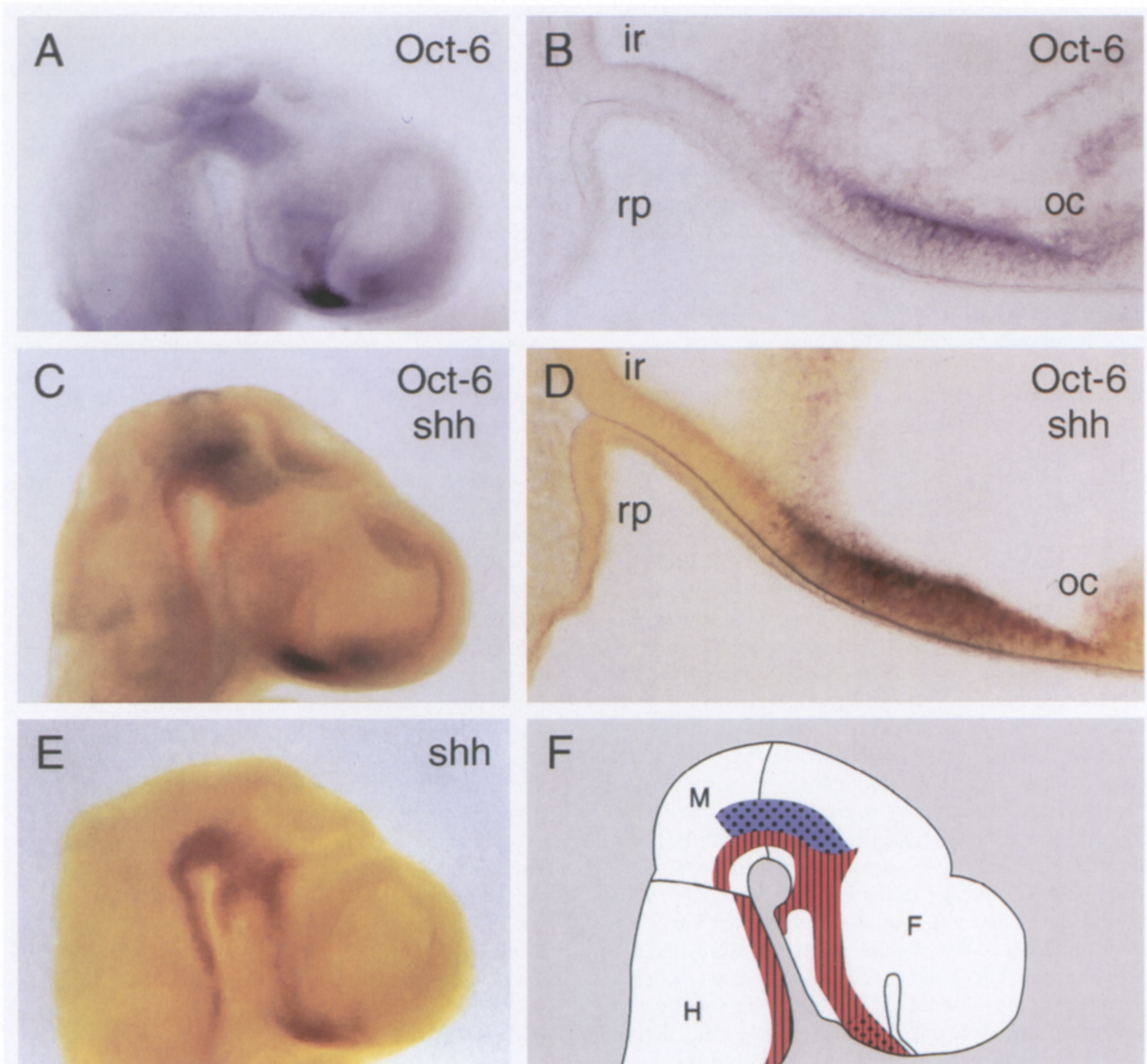

Fig. 6. Oct-6 and Shh in situ hybridization in the developing forebrain. Lateral views of embryos stained for Oct-6 (A), Shh + Oct-6(C) and Shh (E) are shown. (B,D) Midsagittal sections of the embryos depicted in (A) and (C), respectively. Note that Oct-6 expression is contained within the Shh expressing domain at the ventral midline of the anteriormost part of the developing forebrain. The diagram in (F) summarizes the expression patterns of Oct-6 (blue dots) and Shh (red stripes) at the time of rostral neuropore closure. The overlap in expression of Oct-6 and Shh in the rostral diencephalon is indicated with stripes and dots on a red background. Abbreviations are; ir, infundibular recess; rp, Rathke's pouch; oc, optic chiasm.

opmental expression of the gene and the proteins that bind to these elements might give a clue towards the identity of the molecules involved. It is anticipated that negative regulatory elements will play a major role in establishing the expression pattern after the initial widespread activation of the gene.

The high levels of expression of Oct-6 in the ectoderm of the prestreak embryo and in the primitive brain of the advanced headfold stage embryo coincides with a high proliferative activity in these structures. In postnatal Schwann cells high levels of Oct-6 are correlated with proliferation (although this correlation is not a strict one; see Scherer et al., 1994). Recently Faus et al. (1994) described the expression of Oct-6 mRNA in the highly proliferative basal layer of the skin. When cells in this layer start to differentiate and migrate out, Oct-6 expression is downregulated. In support of this, we have observed Oct-6 protein expression in the surface ectoderm and in the

hair follicles of midgestation embryos. High levels of Oct-6 expression in surface ectoderm were especially evident during the formation of the pinnae of the ear and the vibrissae (unpublished observations). It is therefore possible that one function of the Oct-6 gene is in the expansion of undifferentiated ectodermal cells. However, Oct-6 expression has been seen in neurons that have started to differentiate and leave the ventricular zone and in some mature neurons, indicating that the protein might serve an additional function in these cells that is not related to proliferation (Frantz et al., 1994; our unpublished observations). Ectopic expression and gene knock out studies in transgenic mice should reveal if Oct-6 is able to alter the developmental fate of a multi- or totipotent cell type in the neuronal differentiation pathway or influence the differentiation of other ectodermal cells that express Oct-6, like Schwann cells and hair follicle cells.

3.2. Segmental expression of Oct-6 in the mid and forebrain

At the time of anterior neuropore closure Oct-6 expression has adopted a segment-like pattern in the mid- and forebrain (see Fig. 5). The central nervous system of vertebrates overlying the notochord becomes segmented through signaling from the segmented mesoderm (the somites; resulting in a secondary segmentation) or in the hindbrain through a program of intrinsic interactions (Lumsden and Keynes, 1989; Stern et al., 1991). The segmentation of the hindbrain and the associated neural crest has been extensively analyzed by tissue transplantation and gene expression studies. It is clear that the rhombomeres represent genuine domains for cellular differentiation and cell lineage restriction (Keynes et al., 1990). Several developmental control genes like the hox genes and Krox-20 have rhombomere restricted expression patterns and gene knock out experiments in mice resulted in the alteration or absence of the hindbrain structures in which they are expressed (Carpenter et al., 1993; Krumlauf, 1993; Schneider-Manoury et al., 1993; Swiatek and Gridley, 1993).

Whether the mid- and forebrain in the prechordal region also develops through a segmental subdivision is still a matter of debate. Morphological subdivision in the anterior brain region is not as readily apparent as it is in the hindbrain. However, the temporal appearance of furrows in the forebrain has been interpreted in the light of progressive segmentation, defining distinct adult brain structures. Different models have been put forward to describe this subdivision of the anterior brain (for discussion of these different models see Kuhlenbeck (1973) and Puelles and Rubenstein (1993)). The recent description of the restricted expression pattern of a large number of regulatory genes in the forebrain support the notion that the prechordal CNS may also develop through segmentation of the anterior neural tube (Figdor and Stern, 1993; Puelles and Rubenstein, 1993; and references therein). Our immunohistochemistry and RNA in situ data show that Oct-6 expression is also confined to certain domains in the forebrain at the time of rostral neuropore closure. Using Shh as a secondary probe we identified the Oct-6 expression domains as the basal segment of prosomere 1 and 2 in the diencephalon and the ventral part of prosomere 6 of the secondary prosencephalon (Fig. 6F). A number of regulatory genes like pax-6, BF-2, Wnt-3 and Dlx-1 have a similar boundary (caudal or rostral) of expression at the border between prosomere 2/3 which demarcates the zona limitans intrathalamica (Salinas and Nusse, 1992; Hatini et al., 1994; Stoykova and Gruss, 1994) This landmark also corresponds to the caudal limit of neuromere 1 of the diencephalon in the model of Figdor and Stern. It has been speculated that this structure functions as an organizer patterning the adjacent neuroepithelium through planar inductive signals (Rubenstein et al, 1994; Bally-Cuif and Wassef, 1995). It is possible that these inductive signals regulate or restrict the expression of the genes mentioned above, including Oct-6, to a domain caudal or rostral of this boundary.

An organizing or patterning function has also been ascribed to the rostral/ventral region of the forebrain in which Oct-6 and Shh are coexpressed. This region is able to induce, as is COS cell derived SHH, neurons of ventral telencephalic identity in prospective telencephalic neural plate tissue (Ericson, 1995). This suggests that a signal, most likely SHH, emanating from the rostral forebrain is able to pattern the ventral part of the telencephalon. Furthermore, experiments in zebrafish have suggested that ventral midline cells in the forebrain also function in patterning the diencephalon (Hatta, 1994). Ongoing comparative expression studies for Oct-6 in chick and zebrafish and gene knock out experiments in mice will hopefully shed some light on the possible function of the Oct-6 gene in these anterior brain structures.

4. Materials and methods

4.1. Oct-6 overexpression and purification

For overexpression of Oct-6 protein in *E. coli*, a BamHI-BglII fragment from pN1Oct-6, containing the full length Oct-6 cDNA plus the β-globin intron sequences (Meijer et al., 1992), was cloned behind the IPTG inducible T7 promoter in the BamHI site of the pET11A expression vector (Novagen). This construct was transfected into the BL21(DE3). An overnight culture was diluted 1 in 10 and cultured at room temperature to an $OD_{600} = 0.8$. Overexpression was induced by adding IPTG to a final concentration of 0.4 mM and the culture was incubated for 4 h. Pilot experiments have shown that the Oct-6 protein can be purified by high affinity chromatography on a Ni-NTA matrix (Qiagen). Binding of Oct-6 to this matrix is probably mediated by the poly-

histidine stretch present in the carboxyl terminal part of the protein.

For large scale purification, a 500 ml IPTG induced bacteria culture was pelleted, washed once with PBS, resuspended in 10 ml 6 M urea/PBS and sonicated. The cell lysate was cleared by centrifugation at 12 000 rev./min for 5 min at 4°C. Imidazole was added to the supernatant to a final concentration of 0.8 mM and incubated overnight at 4°C with 300 μl Ni-NTA beads (Qiagen). The Ni-NTA was washed twice with 10 ml of a 6 M urea/PBS/0.8 mM imidazole solution for 15 min and three times with 6 M/PBS/8 mM imidazole. Oct-6 protein was eluted from the matrix in 500 μl 6 M urea/ PBS/80 mM imidazole. This purification procedure resulted in high yields of pure (>95%) and intact Oct-6 protein as judged by Coomassie stained SDS-PAAGE.

4.2. Antibodies

The bacterially overexpressed Oct-6 protein was recovered in 6 M urea/PBS/80 mM imidazole. To lower the urea concentration the eluate was dialyzed stepwise against PBS with decreasing concentrations of urea, to a final concentration of 400 mM. This led to the partial precipitation of Oct-6 protein. Antibodies were raised in White New Zealand rabbits by three consecutive injections with 4 weeks intervals of 0.5–1.0 mg Oct-6 protein resuspended in incomplete Freund's adjuvant.

Oct-6 antibodies were affinity purified by binding to Oct-6 protein immobilized on nitrocellulose. After preincubation with 1% BSA/3% powdered milk/0.05% Tween-20/PBS for 2 h at 4°C, the nitrocellulose was incubated overnight with the antiserum that had been precleared with BL21(DE3) cell lysate at room temperature for 3 h. After extensive washing with PBS the Oct-6 antibodies were eluted from the nitrocellulose by a 3 M KSCN/ 0.1 M $NaPO_4$/500 μg/ml BSA solution. To remove the KSCN the antibody solution was passed over a 0.1 M $NaPO_4$ (pH 7.5) equilibrated Sephadex G-50 column.

4.3. Band shift assay

Nuclear protein extracts were made from the Mes68 cell line (Meijer et al., 1990) as described by Schreiber et al. (1989). Band shift assays were performed as described (Meijer et al., 1990).

4.4. Western blot analysis

Cos-1 cells were transiently transfected by the $CaPO_4$ method with 10 μg of the pN1Oct-6 or pN229C52Oct-6 expression vectors encoding the complete Oct-6 protein or the Oct-6 POU domain respectively (Meijer et al., 1992). Seventy-two hours after transfection whole cell extracts were made. Equal amounts of protein were run on a 10% SDS-PAAGE and blotted via semi-dry electroblotting to nitrocellulose filter. The western blot was blocked by overnight incubation at 4°C in a 5% powdered milk/PBS solution. Three 10 min washes in washing buffer (PBS/0.5% Tween20) were followed by 2 h incubation at 4°C with the affinity purified Oct-6 antiserum in PBS/0.5% BSA/0.05% Tween20. After six 10 min washes it was incubated for 2 h with a 1:1000 dilution of the anti-RabbitIgG alkaline phosphatase secondary antibody (Sigma) in PBS/0.5% BSA. After six washes in washing buffer the blot was washed once more in 0.2 M Tris–HCl, (pH 9.0), 10 mM $MgCl_2$. The staining reaction was carried out with the BCIP/NBT substrates.

4.5. Immunohistochemistry

Mouse embryos were derived from natural matings of (CBA × B10)F1 mice. The morning of plugging was determined as 0.5 days post coitum (dpc). Litters of the desired developmental stages were isolated, washed in PBS/0.5% Tween20 (PBS-T) and fixed for 2–4 h in a (35% acetone, 35% methanol, 5% acetic acid and 25% H_2O) solution. After dehydration and paraffin embedding 5 μm sections were made. The sections were mounted on a droplet of 10% alcohol on gelatin/chromesulfate coated Klinipath slides and were allowed to stretch by placing the slides on a hot plate at 42°C. For immunohistochemical staining, the sections were dewaxed and rehydrated. All antibody incubations were done in a 10 mM $NaPO_4$ (pH 7.5) buffer in a horizontally positioned humidified box. The sections were blocked for 2 h by 5% goat serum/0.05% Tween20 and washed 5 times for 10 min in 0.5% Tween20. Incubation with the first antiserum was overnight. To exclude cross reactivity with other closely related class III POU proteins the affinity purified anti-Oct-6 antibodies were preincubated for 1 h with a lysate of Oct-6/POU overexpressing bacteria. As a control, adjacent sections were stained with Oct-6 antibodies and competed with a non-expressing or Oct-6 expressing bacterial lysate. After extensive washing the secondary antibody incubation was performed for 2 h at room temperature. A goat-anti-Rabbit IgG secondary antibody (1:80 dilution) coupled to a FITC chromophore (Nordic) was used to visualize Oct-6 protein expression. After five washes in washing buffer the sections were washed with running tapwater followed by distilled water. The sections were air-dried and embedded in a 100 mg/ml DABCO/ 70% glycerol/PBS solution (Merck). After photographing, the sections were washed in PBS and counterstained with hematoxylin, dehydrated and embedded in entellan (Merck).

In whole mount immunostaining experiments, antibody incubations and washing steps were done in PBSTF (PBS/1% Triton X-100/10% fetal calf serum). Both the primary and secondary antibodies were incubated overnight. As a secondary antibody the alkaline phosphatase conjugated goat-anti-rabbit antibody (Tago) was used.

The staining reaction was carried out with the NBT/BCIP substrates and stopped by washing the embryos in a 50 mM EDTA/PBS solution. The embryos were postfixed in 4% paraformaldehyde/PBS and stored in the dark until photographed.

4.6. Whole mount in situ hybridization

A digoxygenin labeled Oct-6 in situ probe was generated from a 520 bp DraI template which is located in the 3′UTR of the Oct-6 gene. Hybridization and visualization with alkaline phosphatase (AP) coupled DIG antibodies and NBT/BCIP substrates was done as described (Charité et al., 1994). For double labeling experiments the Shh probe (Echelard et al., 1993) was fluorescein labeled and hybridized together with the Oct-6 probe. After staining of Shh with AP-coupled fluorescein antibodies and Fast Red substrate (Boehringer) the embryos were incubated for 10 min in 0.1 M glycine (pH 2.2), followed by the Oct-6 staining reaction (Hauptmann and Gerster, 1994). After postfixation in 4% paraformaldehyde/PBS the embryos were embedded in a phosphate buffered 10% gelatin/30% sucrose solution for cryostat sectioning.

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References

Agarwal, V.R. and Sato, S.M. (1991) Dev. Biol. 147, 363–373.
Ang, S.-L., Conlon, R.A., Jin, O. and Rossant, J. (1994) Development 120, 2979–2989.
Bally-Cuif, L. and Wassef, M. (1995) Curr. Opin. Genet. Dev. 5, 450–458.
Burglin, T., Finney, M., Coulson, A. and Ruvkun, G. (1989) Nature 341, 239–243.
Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R. and Capecchi, M.R. (1993) Development 118, 1063–1075.
Charité, J., de Graaff, W., Shen, S. and Deschamps, J. (1994) Cell 78, 589–601.
Easter, S.S.J., Ross, L.S. and Frankfurter, A. (1993) J. Neurosci. 13, 285–299.
Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A. and McMahon, A.P. (1993) Cell 75, 1417–1430.
Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T.M. and Edlund, T. (1995) Cell 81, 747–756.
Faus, I., Hsu, H.J. and Fuchs, E. (1994) Mol. Cell. Biol. 14, 3263–3275.
Figdor, M.C. and Stern, C.D. (1993) Nature 363, 630–634.
Frantz, G.D., Bohner, A.P., Akers, R.M. and McConnell, S.K. (1994) J. Neurosci. 14, 472–485.
Fukuta, M., Matsuno, K., Hui, C.C. Nagata, T., Takiya, S., Xu, P.X., Ueno, K. and Suzuki, Y. (1993) J. Biol. Chem. 268, 19471–19475.
Hara, Y., Rovescalli, A.C., Kim, Y. and Nirenberg, M. (1992) Proc. Natl. Acad. Sci. USA 89, 3280–3284.
Hatta, K., Püschel, A.W. and Kimmel, C.B. (1994) Proc. Natl. Acad. Sci. USA 91, 2061–2065.
Hatini, V., Tao, W. and Lai, E. (1994) J. Neurobiol. 25, 1293–1309.
Hauptmann, G. and Gerster, T. (1994) Trends Genet. 10, 266.
Hauptmann, G. and Gerster, T. (1995) Mech. Dev. 51, 127–138.
He, X., Treacy, M.N., Simmons, D.M., Ingraham, H.A., Swanson, L.W. and Rosenfeld, M.G. (1989) Nature 340, 35–41.
Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., Ruvkun, G. and Horvitz, H.R. (1988) Genes Dev. 2, 1513–1516.
Hochuli, E., Dobeli, H. and Schacher, A. (1987) J. Chromatogr. 411, 177–184.
Johansen, T., Moens, U., Holm, T., Fjose, A. and Krauss, S. (1993) Nucleic Acids Res. 21, 475–483.
Johnson, W.A. and Hirsh, J. (1990) Nature 343, 467–470.
Keynes, R., Cook, G., Davies, J., Lumsden, A., Norris, W. and Stern, C. (1990) J. Physiol. 84, 27–32.
Krumlauf, R. (1993) Trends Genet. 9, 106–112.
Kuhlenbeck, H. (1973) The Central Nervous System of Vertebrates; Overall Morphologic Pattern, Vol. 3, Part II, Karger, Basel.
Le Moine, C. and Young, III, W.S. (1992) Proc. Natl. Acad. Sci. USA 89, 3285–3289.
Lumsden, A. and Keynes, R. (1989) Nature 337, 424–428.
Meijer, D., Graus, A. and Grosveld, G. (1992) Nucleic Acids Res. 20, 2241–2247.
Meijer, D., Graus, A., Kraay, R., Langeveld, A., Mulder, M.P. and Grosveld, G. (1990) Nucleic Acids Res. 18, 7357–7365.
Monuki, E.S., Kuhn, R. and Lemke, G. (1993) Proc. Natl. Acad. Sci. USA 90, 9978–9982.
Monuki, E.S., Kuhn, R. and Lemke, G. (1993) Mech. Dev. 42, 15–32.
Monuki, E.S., Weinmaster, G., Kuhn, R. and Lemke, G. (1989) Neuron 3, 783–793.
Ninkina, N.N., Stevens, G.E.M., Wood, J.N. and Richardson, W.D. (1993) Nucleic Acids Res. 21, 3175–3182.
Petryniak, B., Staudt, L.M., Postema, C.E., McCormack, W.T. and Thompson, C.B. (1990) Proc. Natl. Acad. Sci. USA 87, 1099–1103.
Puelles, L. and Rubenstein, J.L. (1993) Trends Neurosci. 16, 472–479.
Rosenfeld, M.G. (1991) Genes Dev. 5, 897–907.
Rubenstein, J.L.R., Martinez, S., Shimamura, K. and Puelles, L. (1994) Science 266, 578–580.
Salinas, P.C. and Nusse, R. (1992) Mech. Dev. 39, 151–160.
Scherer, S.S., Wang, D.Y., Kuhn, R., Lemke, G., Wrabetz, L. and Kamholz, J. (1994) J. Neurosci. 14, 1930–1942.
Schneider-Maunoury, S., Topilko, P., Seitanidou, T., Levi, G., Cohen-tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993) Cell 75, 1199–1214.
Schöler, H.R., Hatzopoulos, A.K., Balling, R., Suzuki, N. and Gruss, P. (1989) EMBO J. 8, 2543–2550.
Schöler, H.R., Dressler, G.R., Balling, R., Rohdewold, H. and Gruss, P. (1990) EMBO J. 9, 2185–2195.
Schöler, H.R. (1991) Trends Genet. 7, 323–329.
Snell, G.D. and. Stevens, L.C. (1966) in Green, E.L. (ed.), Biology of the Laboratory Mouse, McGraw-Hill, New York.
Stern, C.D., Jaques, K.F., Lim, T.M., Fraser, S.E. and Keynes, R.J. (1991) Development 113, 239–244.
Stoykova, A. and Gruss, P. (1994) J. Neurosci. 14, 1395–1412.
Suzuki, N., Rohdewohld, H., Neuman, T., Gruss, P. and Schöler, H.R. (1990) EMBO J. 9, 3723–3732.
Swiatek, P.J. and Gridley, T. (1993) Gene Dev. 7, 2071–2084.
Theil, T., McLean, H.S., Zornig, M. and Moroy, T. (1993) Nucleic Acids Res. 21, 5921–5929.
Wegner, M., Drolet, D.W. and Rosenfeld, M.G. (1993) Curr. Opin. Cell Biol. 5, 488–498.