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A role for SOX1 in neural determination

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SUMMARY

In vertebrates, the delineation of the neural plate from a region of the primitive ectoderm is accompanied by the onset of specific gene expression which in turn promotes the formation of the nervous system. Here we show that SOX1, an HMG-box protein related to SRY, is one of the earliest transcription factors to be expressed in ectodermal cells committed to the neural fate: the onset of expression of SOX1 appears to coincide with the induction of neural ectoderm. We demonstrate a role for SOX1 in neural determination and differentiation using an inducible expression P19 cell system as an in vitro model of neurogenesis. Misexpression of SOX1 can substitute for the requirement of retinoic acid to impart neural fate to competent ectodermal P19 cells. Using a series of antigenic markers which identify early neural cell types in combination with BrdU labeling, we demonstrate a temporal and spatial correlation between the differentiation of cell types along the dorsoventral axis of the neural tube and the downregulation of SOX1 expression. SOX1, therefore, defines the dividing neural precursors of the embryonic central nervous system (CNS).

Key words: SOX1, Neurogenesis, Retinoic acid, Mouse, Rat, Neural plate

INTRODUCTION

Development of the vertebrate nervous system begins with the induction of neural plate from a section of ectoderm. Experimental manipulations suggest that in the mouse (Beddington 1994), as in amphibians (Spemann and Mangold, 1924; Spemann, 1938) and chickens (Hamburger, 1988), signals emanating from the organizer (node) induce neural tissue in the adjacent ectodermal epiblast. In the mouse, fate mapping studies have shown that the precursor population of the neural plate is localized to the anterior and distal ectodermal epiblast of the primitive streak-stage embryo (Tam, 1989; Lawson et al., 1991; Quinlan et al., 1995; Tam and Zhou, 1996). Once induced to become neural, this population of cells undergoes a rapid expansion (Snow, 1977; Poelmann, 1980; Tam et al., 1993) and a combination of epigenetic and genetic patterning mechanisms act on it to specify regional fate and cell identity to generate distinct cell types along the future anteroposterior and mediolateral axes of the neural plate (reviewed by McKay, 1989; Anderson, 1993; Ruiz i Altaba, 1994; Tanabe and Jessell, 1996; Lumsden and Krumlauf, 1996).

The molecular mechanisms controlling neural induction and determination have begun to be elucidated. The identification, by cellular and biochemical methods, of secreted molecules involved in neural induction (noggin, follistatin, chordin) illustrate the important role of the environment in specifying cell identity (reviewed by Harland, 1997). In addition, a number of transcription factors have been isolated which play important roles in the specification and differentiation of neural cell lineages. For example, the characterization of vertebrate homologues of *Drosophila* proneural and neurogenic genes, which control neural specification in the fly, has revealed analogous molecular mechanisms in vertebrate neural cell fate determination and differentiation. In *Drosophila*, the expression of basic helix-loop-helix transcription factors of the Achaete-Scute (AS-C) complex confers neural potential on groups of ectodermal cells. Mutations in genes of the AS-C complex results in a decrease in the number of neuroblasts and conversely the misexpression of these genes leads to an increase in neuroblast number (reviewed by Campos-Ortega, 1993). In a similar fashion, the misexpression of vertebrate basic helix-loop-helix proteins in *Xenopus* embryos, such as XASH-3 (Ferreiro et al., 1994; Turner and Weintraub, 1994; Chitnis and Kintner, 1996), CASH-4 (Henrique et al., 1997), Neurogenin (Ma et al., 1996), NeuroD (Lee et al., 1995) and ATH-3 (Takebayashi et al., 1997), results in either the expansion of the neural plate or an increase in the number of primary neurons. This suggests that basic helix-loop-helix proteins may play vital roles in the acquisition of neural identity. However, in the mouse, the time of onset and restricted patterns of expression of reported bHLH proteins (Lee et al., 1995; Ma et al., 1996; Takebayashi et al., 1997) precludes them from a role in neural determination.

SOX proteins constitute a family of transcription factors related to the mammalian testis determining factor SRY through homology within their HMG-box DNA binding

domains (Gubbay et al., 1990; Sinclair et al., 1990; Denny et al., 1992; Wright et al., 1992; Coriat et al., 1993). In DNA binding studies, SOX proteins exhibit sequence-specific binding (Harley et al., 1994); however, unlike most transcription factors, binding occurs in the minor groove resulting in the induction of a dramatic bend within the DNA helix (Ferrari et al., 1992; Giese and Grosschedl, 1992; Van de Wetering et al., 1992; Werner et al., 1995; Love et al., 1995). Although SOX proteins can induce transcription of reporter constructs in vitro and possess activation domains (van de Wetering et al., 1993; Hosking et al., 1995; Kamachi et al., 1995; Kanai et al., 1996; Sudbeck et al., 1996), transcriptional activation by these factors appears to be context-dependent (Kamachi et al., 1995; Yuan et al., 1995). In other words members of this family seem to act in conjunction with other proteins. Therefore, SOX proteins display properties of both classical transcription factors and architectural components of chromatin (reviewed by Pevny and Lovell-Badge, 1997).

Members of the *Sox* gene family are expressed in a variety of embryonic and adult tissues, where they appear to be responsible for the development and/or elaboration of particular cell lineages. *Sry* is transiently expressed in the precursor Sertoli cells of the XY genital ridge and is responsible for triggering development of the male phenotype (reviewed by Lovell-Badge and Hacker, 1995) thus, the lack of *Sry* results in XY females and the misexpression of *Sry* results in XX males. *Sox9* is expressed in immature chondrocytes and male gonads (Wright et al., 1995; Morais-da-Silva et al., 1996; Kent et al., 1996; Ng et al., 1997); mutations in the human SOX9 gene are associated with Campomelic Dysplasia, a human skeletal malformation syndrome, and XY female sex reversal (Foster et al., 1994; Kwok et al., 1995; Sudbeck et al., 1996). *Sox4* is expressed in many tissues (Schillam et al., 1993; van de Wetering et al., 1993) and a null mutation of the gene in mouse results in the absence of mature B cells and heart malformations (Schillam et al., 1996). Xsox17 genes are involved in endoderm formation in *Xenopus* embryos (Hudson et al., 1997). These functional analyses suggest that *Sox* genes function in cell fate decisions in diverse developmental pathways.

A subfamily of *Sox* genes, which includes *Sox1*, *Sox2* and *Sox3*, show expression profiles during vertebrate embryogenesis that suggest the genes could function in the control of cell fate decisions within the early developing nervous system (Collignon, 1992; Uwangho et al., 1995; Collignon et al., 1996; Streit et al., 1997, Rex, 1997). *Sox2* and *Sox3* begin to be expressed at preimplantation and epiblast stages respectively (Collignon et al., 1996; Parsons, M., Pevny, L., and Lovell-Badge, R., unpublished data), and are then restricted to the neuroepithelium. *Sox1* appears only at around the stage of neural induction (Collignon, 1992). To address whether this subfamily of genes may play a role in controlling neural determination, we focus here on the expression profile and function of SOX1. We show that the onset of SOX1 expression correlates with the formation of neural plate. The onset of SOX1 expression in P19 embryonal carcinoma cells is similarly dependent on neural induction. Thus, SOX1 appears to be an early response to neural inducing signals both in vivo and in vitro. Expression of SOX1 within the CNS is transient and associated with dividing neural cells. Using an inducible promoter system we show that the upregulation of SOX1 expression is itself sufficient to impart neural fate on competent ectodermal P19 cells, suggesting that expression of this transcription factor may normally operate in vivo to bias cells to a neural fate.

MATERIALS AND METHODS

Manufacture of SOX1 polyclonal antibodies

A 622 bp *Hinc*II fragment encoding sequences C-terminal to the HMG box of SOX1 (207 aa) was fused in frame to the bacterial GST gene in the construct pGEX3X. Fusion protein was induced and purified as described by Smith and Johnson (1988). Rabbits were treated with a course of injections as recommended by Smith and Johnson (1988): each injection contained 250 μg of fusion protein. Two final bleeds, FB43 and FB44, were obtained from the rabbits prior to the preparation of polyclonal sera.

Immunocytochemistry

Embryos, P19 cells and neural plate explants were examined using standard techniques (Placzek et al., 1993). Antibodies were used at the following dilutions: anti-SOX1 pAb (1:500); K2 anti-HNF3β mAb (1:40); 6G3 anti-FP3 mAb (1:10); anti-3A10 mAb (1:10); anti-2H3 (Neurofilament-160) mAb (1:10); 4D5 anti-Islet-1 mAb (1:100); anti-SSEA1 mAb (1:80) (Hybridoma Bank); anti-NESTIN mAb (1:10) (Hybridoma Bank); anti-BrdU mAb (1:500) (Sigma); appropriate secondary antibodies (TAGO and Sigma) were conjugated to fluorescein isothiocyanate (FITC), Cy2, or Cy3.

BrdU analysis

Pregnant mice (Parkes outbred strain) were injected intraperitoneally with 50 μg/g of body weight of 5-bromo-2-deoxyuridine (BrdU) (Sigma) in 0.9% NaCl and killed 2 hours after injection. Embryos were fixed and sectioned as described above. The slides were washed twice in PBS, and incubated in 0.2% HCl at 37°C for 30 minutes, then rinsed thoroughly with PBS, followed by three rinses with PBS/0.1% Triton X-100/1% heat inactivated goat serum (P-T-G). Monoclonal anti-BrdU (1:500 dilution in P-T-G) was applied to the sections and incubated at 4°C overnight. Sequential sections were incubated in SOX1 antibody (1:500 dilution in P-T-G) at 4°C overnight. The slides were washed twice in P-T-G, then incubated in the appropriate secondary antibody for 30 minutes at room temperature, washed with P-T-G and mounted.

P19 cell culture and retinoic acid treatment

P19 cells were cultured as previously described (Rudnicky and McBurney, 1987). To induce differentiation, cells were allowed to aggregate in bacterial grade Petri dishes alone, in the presence of 1 μM retinoic acid or in the presence of 5 mM IPTG (isopropyl β-D thiogalactoside). After 4 days of aggregation in the presence of inducing agents, cells were plated on tissue culture chamber slides. The cells were then allowed to adhere and grow for 4-5 days, with media changes every 24 hours. For immunofluorescence, cells were grown on tissue culture chamber slides coated with 0.1% gelatin, washed once with PBS, fixed at room temperature in 1× MEMFA for 1 hour, washed in P-T-G twice; then stained with the appropriate antibody.

Cell counting

For cell counting experiments P19 transfectant cell lines were induced to differentiate (as above) plated on gelatin-coated slides, cultured 6-8 days to allow neuronal differentiation fixed at room temperature in 1× MEMFA for 1 hour. Cells were stained with Neurofilament (2H3) antibody and photographed using an Olympus fluorescence microscope. Cell counts are expressed as percentages of total cells in a field. Eight fields from two different experiments were counted for each P19 clone.

Plasmids and transfection

To construct the SOX1 expression vector, pRSVopSox1, the POP113CAT operator vector (Stratagene) was digested with *Not*1, end-filled with Klenow, and the 5467 bp fragment was gel isolated and ligated to an end-filled *Kpn*I/*Stu*I (position 431-1694) fragment of the *Sox1* cDNA. The P3′SS, eukaryotic Lac repressor expressing vector (obtained from Stratagene) was transfected into P19 cells by lipofection. Stable transformants were selected in 250 μg/ml of hygromycin. Expanded clones (250) were isolated and examined for expression of the Lac repressor by indirect immunofluorescence with anti-lac pAb (Stratagene). Four cell lines were isolated P3′SS-10, 13, 22, 47, which showed ubiquitous and constitutive expression of the Lac repressor. P3′SS-10 was chosen for the subsequent experiments. P3'SS-10 was then transfected with pRSVopSox1 by lipofection. Stable clones were selected using 500 μg/ml G418. 250 clones were expanded and analyzed for inducible *Sox1* expression by RNase protection and immunocytochemistry with the SOX1 antibody.

RNase protection assays

Total RNA was prepared from P19 cells and RNase protection assays were carried out using 5 μg of P19 cell RNA as described by Capel et al. (1993). Anti-sense labeled probes were derived from the 396 bp *Sma*I-*Bsp*H1 fragment (position 1467-1863) of the *Sox1* cDNA, a 215 bp *Bsa*I exon 4 specific fragment of the *Wnt1* cDNA, a *Pvu*II digest of the *Mash1* cDNA (Johnson et al., 1992) and a *Not*I digest of SAP D cDNA was used as a loading control (Dresser et al., 1995).

RT-PCR

Total RNA was prepared from P19 cells as described by Capel et al. (1993). Reverse transcription, PCR reaction, and primers was performed as described by Okabe et al. (1996).

Rat lateral neural plate explants

Lateral neural plates (LNP) were isolated from prospective hindbrain and spinal cord regions of day 8.5-9.0 rat embryos, as previously described (Placzek et al., 1993). Notochord explants were dissected from HH stage 6-8 chick embryos as previously described (Placzek et al., 1993). Explants were embedded in collagen and cultured (Placzek et al., 1993) for 24, 48 and 96 hours. Purified rat SHH-N (Ericson et al., 1996) was added to cultures at concentrations within the effective ranges used in other assays (Ericson et al., 1996).

RESULTS

Expression of SOX1 during early neural development

SOX1 expression during mouse and rat neurulation was analyzed using a rabbit polyclonal antibody against the SOX1 C-terminal region. In the mouse, expression of SOX1 is first detected at 7.5 days post coitum (*dpc*) in the anterior half of the late-streak egg cylinder. Cross sections through the embryo at this stage reveal expression in columnar ectodermal cells, which appear to define the neural plate, while cells located more laterally are negative (Fig. 1A). SOX1 is maintained in all neuroepithelial cells along the entire anteroposterior axis as the neural plate bends (8.0-8.5 dpc, Fig. 1B) and fuses to form the neural tube (9.0-9.5 dpc; Fig. 1C). The pattern of expression of SOX1 in the rat is similar to that in the mouse (data not shown). The expression of SOX1 throughout the neural plate and early neural tube implies a similarity amongst these cells.

After neural tube closure, neuroepithelial cells begin to differentiate into defined classes of neurons at specific dorsoventral (D/V) positions within the spinal cord (Altman and Bayer 1984; Tanabe and Jessell, 1996). As development proceeds, SOX1 is downregulated in a stereotyped manner in cells along the D/V axis of the neural tube. In the spinal cord,

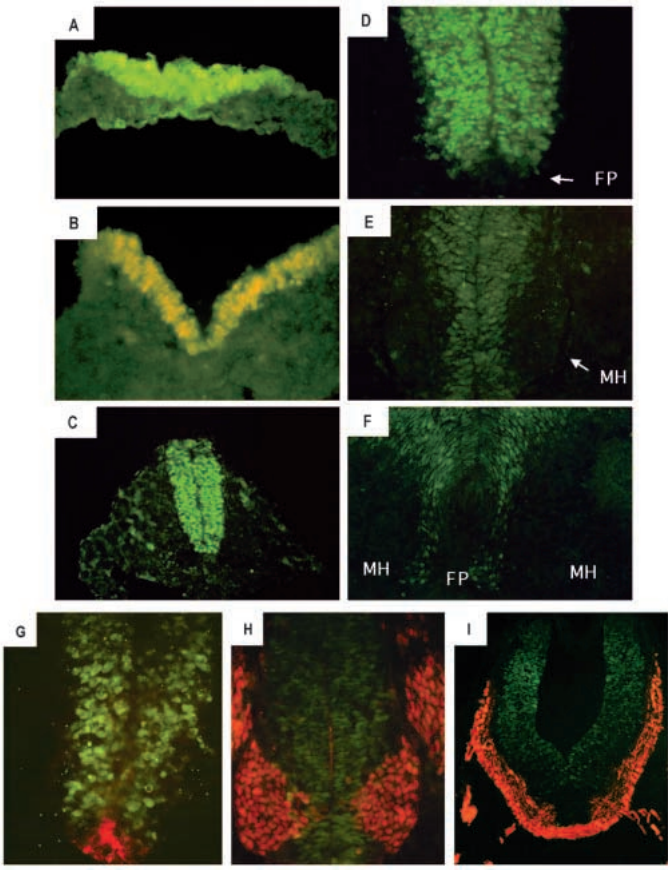

Fig. 1. Immunohistochemical analysis of SOX1 during mouse and rat embryogenesis. (A) Transverse section through the anterior region of a 7.5 *dpc* mouse embryo. SOX1 labeling is restricted to the neural plate. (B) Transverse section through the anterior region of a 2 somite mouse embryo. SOX1 labeling is restricted to the neural folds. (C) Transverse section through the posterior region of a 10-12 somite mouse embryo. SOX1 labeling is restricted to the neural tube. (D) Transverse section through the thoracic region of a 20 somite mouse embryo. SOX1 labeling is detected in the early neural tube but is excluded from the ventral midline region of the floor plate (FP, arrow). (E) Transverse section through the thoracic region of a 30-35 somite mouse embryo. SOX1 labeling is not detected in the ventral motor horns (MH). (F) Transverse section through the anterior spinal cord region of a 30-35 somite mouse embryo. Expression of SOX1 is maintained in 'region X', bilateral streams of cells between the floor plate (FP) and motor horns (MH). (G) Expression of SOX1 (green) and FP3 (red) in a transverse section through the thoracic region of an E10 rat embryo. Expression of SOX1 and FP3 are mutually exclusive. (H) Expression of SOX1 (green) and Islet1 (red) in a transverse section through the thoracic region of an E11 rat embryo. Expression of SOX1 and Islet1 are almost mutually exclusive although there appears to be some overlap in the most medial differentiating Islet1-positive cells. (I) Expression of SOX1 (green) and Neurofilament (red) in a transverse section through the thoracic region of an E11 rat embryo. Expression of SOX1 and Neurofilament are mutually exclusive.

expression is first downregulated in cells that occupy the ventral midline (Fig. 1D), then the ventral motor horns (Fig. 1E) and subsequently the dorsal regions (not shown). These regions appear to correlate with floor plate, motor neurons and sensory relay interneurons, respectively. To ascertain this we performed a series of antibody double-labeling experiments in rat embryos. The SOX1 antibody was used in combination with a panel of antigenic markers which identify cells of the floor plate (FP3: Fig. 1G), motor neurons (Islet1: Fig. 1H), sensory relay interneurons (TAG1: not shown) and mature neurons (Neurofilament (NF-1); Fig. 1I). As illustrated by these examples, expression of SOX1 and expression of these markers is almost entirely mutually exclusive. In the ventral spinal cord of the 10.0-12.0 *dpc* mouse embryo, SOX1 expression is maintained only in 'region X' (Yamada et al., 1991), as revealed by immunolabeling of two streams of cells located between the differentiated floor plate and ventral motor horns (Fig. 1F and see below). Eventually, by 13.5 *dpc,* SOX1 expression is restricted to a thin ventricular zone in the CNS (data not shown). SOX1 expression is not detected in the peripheral nervous system (PNS). These expression profiles suggest that SOX1 is expressed by early neural cells in the CNS and is downregulated in the developing neural tube coincident with neural differentiation.

SOX1 marks proliferating cells within the embryonic neural tube

The uniform expression of SOX1 in the neural plate and early neural tube followed by its downregulation along the D/V axis and restriction to the ventricular zone is reminiscent of the pattern of cell proliferation in the developing central nervous system (Sauer, 1935; Fujita, 1963; Altman and Bayer, 1984). In the neural plate and early neural tube, proliferating progenitor cells are organized in a pseudostratified epithelium in which the processes of these cells extend from the inner luminal to the outer mantle surface. At later stages the neural tube becomes progressively thicker and can be divided into different zones. The proliferating CNS progenitors are largely restricted to the inner ventricular zone (VZ) around the lumen. They begin to migrate away from the lumen while in S-phase, and after completing their final mitosis, migrate to the outer layer, the marginal zone (MZ). In the 10.5 dpc mouse embryo, SOX1 expression is detected throughout the pseudostratified epithelium of the posterior neural tube (Fig. 2A) and is restricted to the ventricular zone in more mature anterior region of the neural tube (Fig. 2C,E). In order to evaluate the relationship between SOX1 expression and proliferating CNS cells we directly assayed proliferation by monitoring the incorporation of bromodeoxyuridine (BrdU). Pregnant female mice at 10.5 dpc were injected with BrdU 2 hours prior to dissection to detect proliferating cells. Embryos were then fixed, sectioned and double-labeled for BrdU incorporation and SOX1 expression. Similar to SOX1-expressing cells, those that incorporate BrdU are found throughout the posterior neural tube (Fig. 2B) and lie in the ventricular zone of the anterior neural tube (Fig. 2D,E). As shown in Fig. 2E-G, all cells that incorporated BrdU also expressed SOX1. SOX1-positive cells that did not incorporate BrdU are restricted to the luminar surface of the ventricular zone (Fig. 2G, small arrow). In contrast, no SOX1- or BrdU-positive cells were detected in the outer marginal zone (Fig. 2G, large arrow). These results suggest that SOX1 is expressed in dividing neuroepithelial cells within the embryonic CNS.

Downregulation of SOX1 in the neural tube correlates with exit from mitosis

The mutual exclusion of SOX1 and markers of committed differentiated cells such as Islet1 (Pfaff et al., 1996) raises the possibility that the downregulation of SOX1 may be a pre-requisite step for the differentiation of neural cells. To address this we monitored cell differentiation in neural plate explants in vitro. Isolated neural plate explants were cultured with known inducers of ventral neural cells, namely the notochord and purified Sonic Hedgehog protein. The expression of SOX1 and incorporation of BrdU was then compared to the expression of three markers of ventral cells, Islet1, FP3 and HNF3β. Consistent

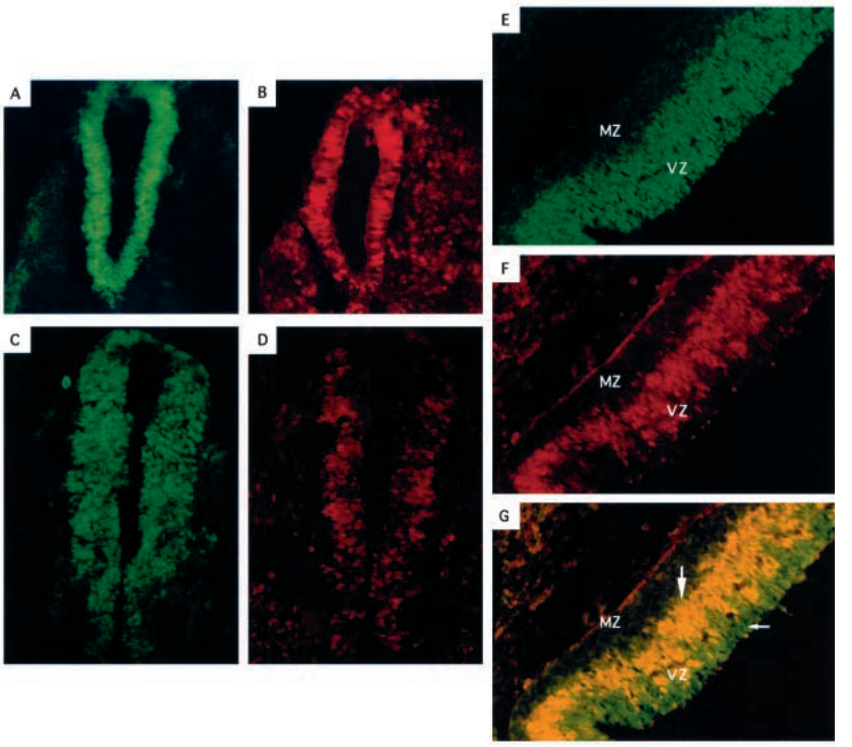

Fig. 2. SOX-1 is expressed in mitotically active (BrdU+) cells within the embryonic neural tube. Transverse sections through 10.5 dpc mouse embryo stained with SOX1 PAb (green) and BrdU MAb (red). (A,B) Serial transverse sections from hindlimb region of 10.5 dpc mouse embryo stained with anti-SOX1(A) and anti-BrdU(B) antibodies. (C,D) Serial transverse sections from forelimb region stained with anti-SOX1(C) and anti-BrdU (D) antibodies. (E-G) Transverse section from midbrain region of 10.5 dpc mouse embryo double labeled for SOX1 (E) and BrdU (F). (G) Superimposition of both staining patterns. Small arrow indicates the restriction of BrdU to the luminar surface of the ventricular zone (VZ) and the large arrow indicates the absence of SOX1 and BrdU in the outer marginal zone (MZ).

with our observations in vivo (Fig. 1G,H) both the expression of SOX1 and Islet1 as well as SOX1 and FP3 was mutually exclusive in neural plate explants cultured adjacent to notochord (*n*=8, data not shown) or in the presence of purified Sonic Hedgehog protein (Fig. 3A-F). Similarly, the incorporation of both BrdU and Islet1 (data not shown) as well as BrdU and FP3 (Fig. 3G-H) was mutually exclusive. In contrast, the domain of expression of HNF3β was found to extend beyond that of FP3 and into the region of BrdU-positive cells (Fig. 3G-I).

To determine whether a similar population of cells could be detected in vivo, embryos were analyzed for co-expression of FP3 and HNF3β, and for co-expression of BrdU and HNF3β. We found that medial floor plate cells co-express HNF3β and FP3 but do not incorporate BrdU (Fig. 3J-N), whereas lateral floor plate cells express only HNF3β and incorporate BrdU (Fig. 3M,N). HNF3β thus provides a marker for cells that are mitotically active but have begun to differentiate. We therefore asked whether these cells express SOX1 or whether expression is already lost from these cells. As shown in Fig. 3O-Q, cells occupying the medial regions of the floor plate express HNF3β but not SOX1. In contrast cells occupying lateral regions of the floor plate co-express HNF3β and SOX1. These observations, together with the mutually exclusive expression of SOX1 with Islet1 and FP3 in ventral neural cells, provide evidence that SOX1 is downregulated as cells exit mitosis and not at the onset of cell differentiation.

P19 EC cells: induction of SOX1 expression upon neural differentiation

Neural induction is accompanied by the onset of new gene

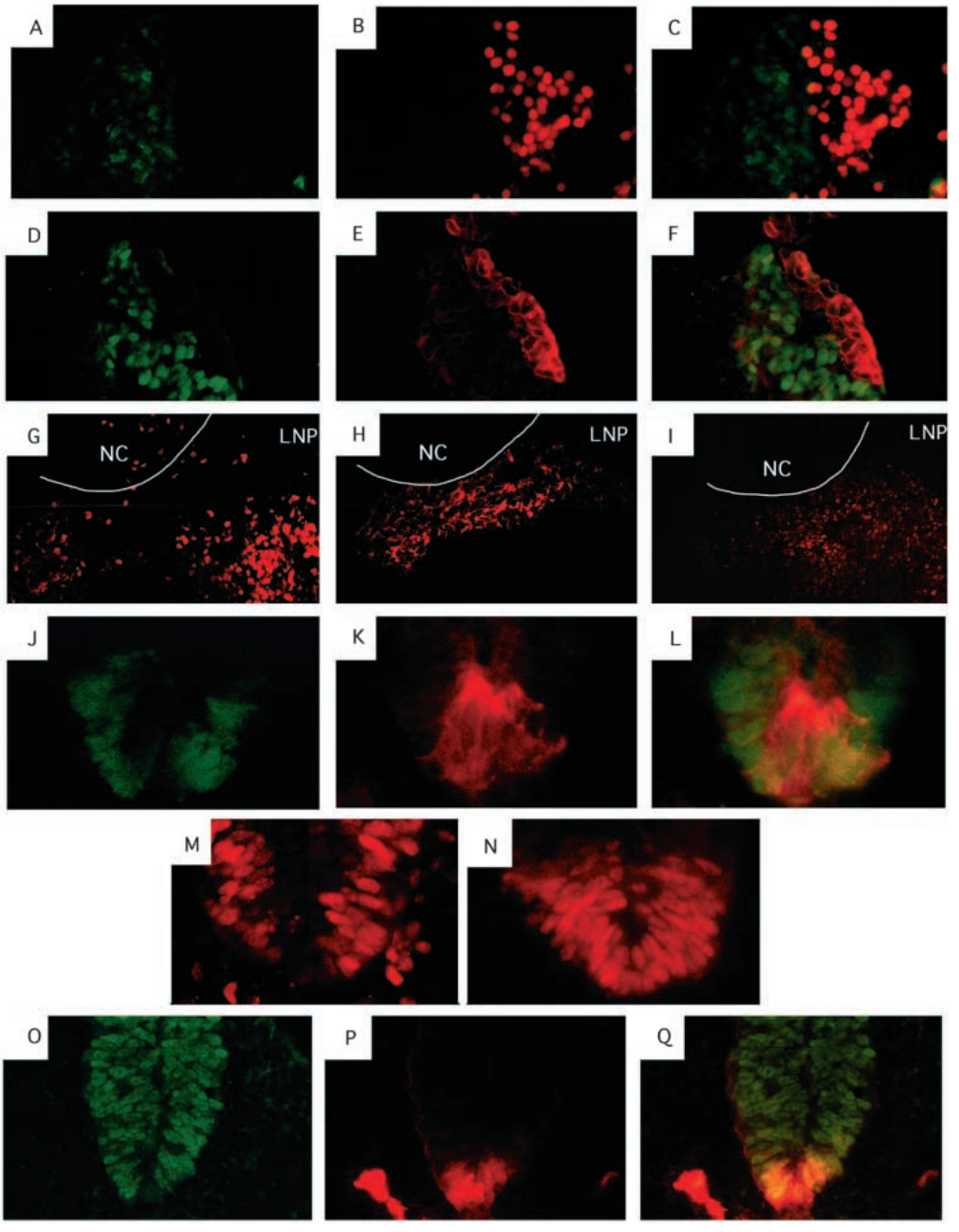

Fig. 3. Downregulation of SOX1 in ventralized lateral neural plate. (A-C) E9 rat lateral neural plate (LNP) cultured with purified SHH protein for 48 hours and stained with anti-SOX1 (A), anti-Islet1 (B), and double labeled with anti-SOX1 and anti-Islet1 (C) antibodies. Expression of SOX1 and Islet1 are mutually exclusive. *n*=7. (D-F) E9 rat neural plate cultured with purified SHH protein for 48 hours and stained with anti-SOX1 (D), anti-FP3 (E) and double labeled with anti-SOX1 and anti-FP3 (F) antibodies. Expression of SOX1 and FP3 are mutually exclusive. *n*=8. (G-I) E9 rat lateral neural plate cultured adjacent to a HH st. 6 chick notochord for 48 hours, pulse labeled with BrdU for 2 hours and stained with anti-BrdU (G), anti-FP3 (H) and anti-HNF3β (I) antibodies. *n*=6. (J-L) Transverse section through ventral midline of E9 rat thoracic neural tube stained with anti-HNF3β (J), anti-FP3 (K) and double labeled with anti-FP3 and anti-HNF3β antibodies (L). (M,N) Transverse section through a thoracic region of a 20 somite mouse neural tube stained with anti-BrdU (M) and anti-HNF3β antibodies (N). (O-Q) Transverse section through a thoracic region of E9 rat neural tube stained with anti-SOX1 (O), anti-HNF3β (P) and double labeled with anti-SOX1 and anti-HNF3β (Q) antibodies. NC, notochord.

expression which in turn enables the formation of neural rather then epidermal tissue. The early and apparently uniform expression of SOX1 in neural cells, together with observations that *Sox* genes may affect cell lineage decisions (see Introduction), raises the possibility that SOX1 expression is an early response to neural inducing signals and that its expression may be involved in directing cells towards a neural fate. To begin to address whether SOX1 plays a role in establishing neural fate in response to inducing signals we have used the P19 cell culture system as an in vitro model system in which to analyze SOX1 expression and the effects of its misexpression.

P19 cells are an embryonal carcinoma cell line with the ability to differentiate into all three germ layers (McBurney, 1993). In the undifferentiated state P19 cells morphologically resemble an uncommitted primitive ectodermal cell and express the cell surface antigen SSEA-1. These cells have a very low rate of spontaneous differentiation when grown in a monolayer in the absence of chemical inducers. P19 cells grown as aggregates, however, differentiate partially into endodermal cells. Furthermore, with the addition of retinoic acid, aggregated P19 cells differentiate into neuroepithelial-like cells (Jone-Villeneuve et al., 1982) These express neuroepithelial markers such as NCAM, intermediate filament NESTIN, MASH1 (Johnson et al., 1992), and WNT1 (St. Arnaud et al., 1989). When plated onto a substrate, about 15% of these cells differentiate into mature neurons expressing Neurofilament. Thus, in this in vitro model system retinoic acid acts as a 'neural inducer'.

Initially, we examined the expression of *Sox1* in P19 cells by both RNase protection and immunocytochemistry. The features of *Sox1* expression in P19 cells are similar to those observed in prospective neural tissue in vivo. *Sox1* mRNA and protein could not be detected in undifferentiated P19 cells which express the cell-surface antigen SSEA1 (Figs 4A,B, 5A). Similarly, when P19 cells are differentiated as aggregates without the addition of chemical inducers, SOX1 is not expressed (Fig. 5A). In contrast, SOX1 is rapidly induced during neural differentiation when aggregated P19 cells are differentiated in the presence of retinoic acid (Fig. 5A). *Sox1* thus behaves similarly to other neuroepithelial markers such as *Mash1* and *Wnt1* (Fig. 5A). When retinoic acid-treated P19 cell aggregates are plated onto tissue culture substrate, about 15% of the cells differentiate into mature process-bearing, Neurofilament-expressing neurons. To examine the expression of SOX1 in P19 cells displaying a fully differentiated neuronal morphology we used double-label immunofluorescence to simultaneously detect SOX1 and Neurofilament. As shown in Fig. 4C-F, SOX1 immunoreactivity was not detected in process-bearing Neurofilament-positive neurons. Thus, as in vivo, SOX1 is expressed by P19 cells when they first assume a neural fate but it is then downregulated with their differentiation.

SOX1 directs p19 cells to a neural fate

The previous data suggest that in P19 cells, as in vivo, SOX1 expression is induced at a time when neuroepithelial cells begin to differentiate. If SOX1 plays a role in directing cells towards the neural fate, we might expect that expression of SOX1 in P19 cells could substitute for retinoic acid to initiate neural differentiation. We therefore designed a way to activate SOX1 in P19 cells using an inducible eukaryotic lac repressor-operator expression system. To establish this system a clonal line of P19 cells was generated which constitutively and ubiquitously expresses the lac repressor. This parent line (P3′SS-10) was transfected with pRSVopSox1, a vector containing the *Sox1* cDNA under the regulation of an inducible RSV promoter and stable lines were established. In the uninduced state, without the addition of isopropyl-β-d-thiogalactase (-IPTG) these lines express high levels of the lac repressor that binds to operon sites upstream of the RSV promoter and thus blocks transcription of *Sox1* (Fig. 3B). Upon addition of IPTG (+IPTG) a conformational change occurs, decreasing the affinity of the repressor and resulting in the activation of pRSVopSox1. Approximately 250 clones of transfectants were isolated in the repressed state. Using RNase protection and immunocytochemistry assays we selected three

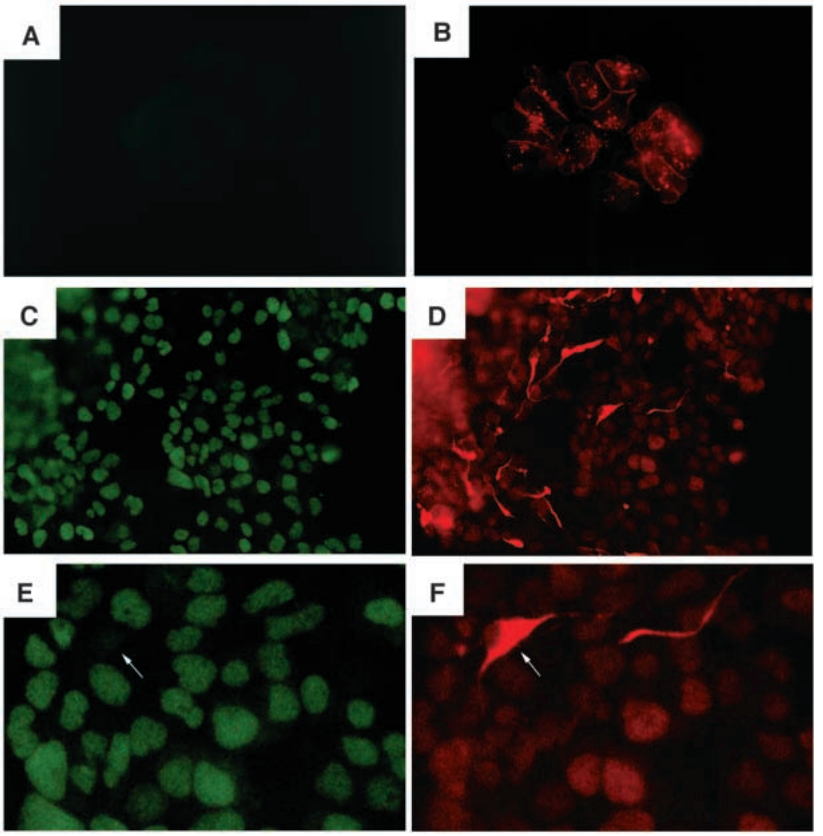

Fig. 4. Immunohistochemical analysis of SOX1 in P19 cells. (A) Undifferentiated P19 cells labeled with anti-SOX1 antibodies. No SOX1 staining is detected. (B) Undifferentiated P19 cells labeled with anti-SSEA1 antibodies. (C,E) P19 cells treated with retinoic acid in suspension for 96 hours subsequently plated onto substrate and labeled with anti-SOX1 antibody after 48 hours. (D,F) P19 cells treated with retinoic acid in suspension for 96 hours, subsequently plated onto substrate and labeled with anti-Neurofilament antibody after 48 hours. Arrows indicate a Neurofilament-positive cell which does not express SOX1.

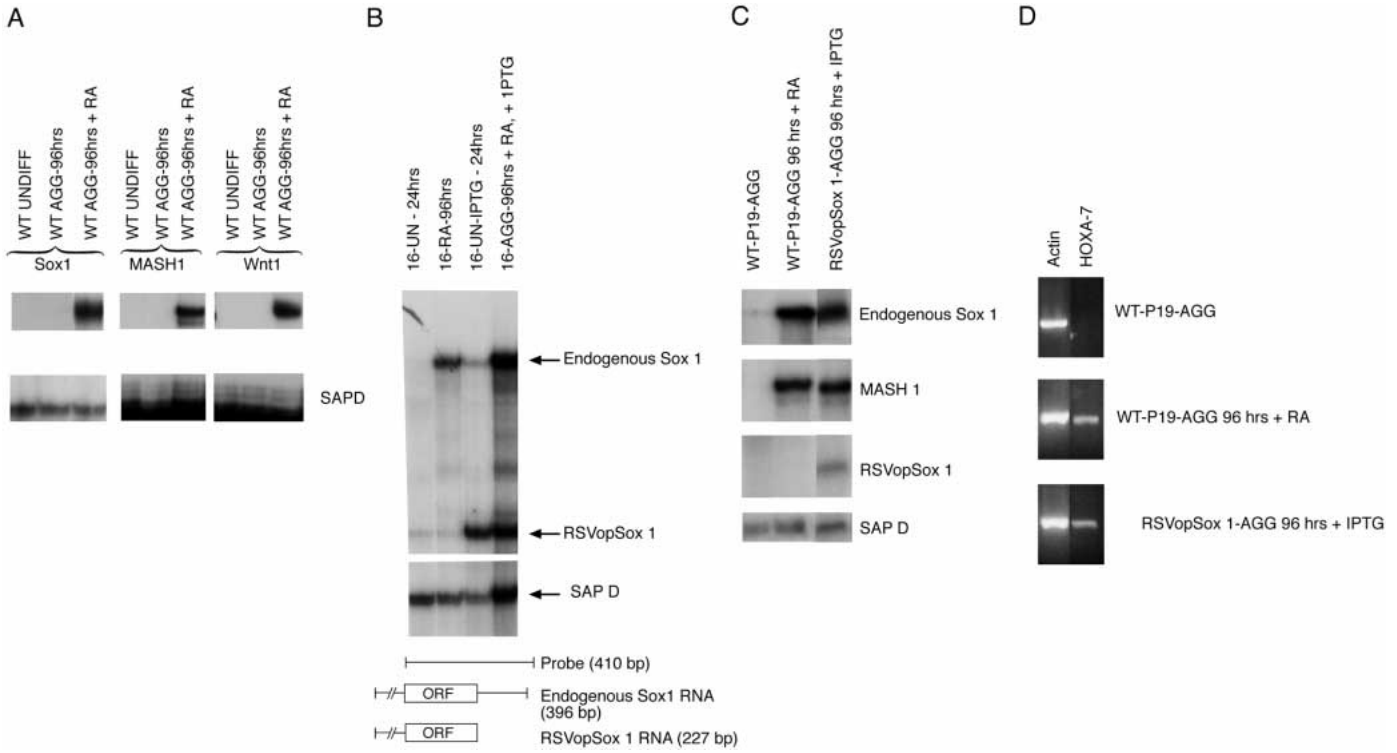

Fig. 5. RNase protection and RT-PCR analysis of *Sox1* expression in P19 cells. (A) Expression of *Sox1* RNA, *Mash1* RNA and *Wnt1* RNA in differentiating P19 cells. *Sox1*, *Mash1* and *Wnt1* transcripts are evident in retinoic acid-treated P19 aggregates (WT AGG-96hrs +RA) but not detected in undifferentiated P19 cells (WT UNDIFF) and P19 aggregates (WT AGG-96hrs). (B) RNA expression analysis of P19 RSVopSox1 transformant (clone 708-16) before and after induction with IPTG. Undifferentiated 708-16 cells (16-UN) do not express endogenous *Sox1* and only a very low level of RSVopSox1 RNA. 708-16 cells treated as aggregates with retinoic acid for 96 hours (16-RA-96hrs) express endogenous *Sox1* and a very low level of RSVopSox1. 708-16 cells treated with IPTG for 24 hours (16-UN-IPTG) express RSVopSox1 and a low level of endogenous *Sox1*. 708-16 cells treated as aggregates with both retinoic acid and IPTG express both endogenous *Sox1* and RSVopSox1. (C) Comparison of the expression of *Sox1* and *Mash1* between wild-type P19 cells grown as aggregates alone (WT-P19-AGG), grown as aggregates treated with only retinoic acid (WT-P19-AGG +RA) and clone 708-16 grown as aggregates and induced with IPTG (RSVopSox1-AGG + IPTG). Wild-type P19 cells treated with retinoic acid and 708-16 cells induced with IPTG express both *Sox1* and *Mash1*. (D) RT-PCR analysis of wild-type P19 cells grown as aggregates alone (WT-P19-AGG), grown as aggregates treated with only retinoic acid (WT-P19-AGG +RA) and clone 708-16 grown as aggregates and induced with IPTG (RSVopSox1-AGG + IPTG).

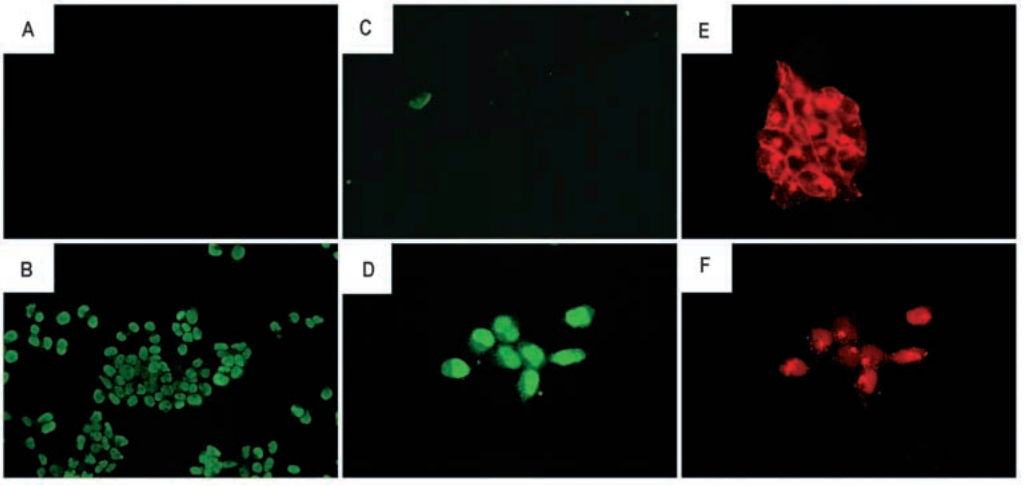

Fig. 6. Immunohistochemical analysis of the P19-RSVopSox1 transformant (clone 708-16). (A,C) 708-16 cells grown as monolayers without the addition of IPTG and stained with the SOX1 PAb. No SOX1 expression is detected. (B,D) 708-16 cells grown as monolayers for 24 hours in the presence of IPTG and stained with the SOX1 antibody. Expression of SOX1 is detected in the majority of the cells (B) and it is localized to the nucleus (D). (E,F) 708-16 cells grown as monolayers for 24 hours without IPTG (E) and with IPTG (F) and stained with anti-SSEA-1 antibody. SSEA-1 is expressed in uninduced 708-16 cells (E).

clones (708-13, 708-16 and 708-21) that expressed high levels of RSVopSox1 in response to IPTG (Fig. 5B lane 3; see also Fig. 6A-D). We next determined that the pluripotentiality of these clones was not compromised by the transfection and selection. All three lines expressed SSEA1 in the uninduced state (Fig. 6E and data not shown). Furthermore, when aggregated in the presence of retinoic acid the uninduced clones initiated expression of endogenous *Sox1* (Fig. 5B, lane 2) and differentiated into mature Neurofilament-expressing neurons after plating (data not shown), in a manner similar to wild-type P19 untransfected cells.

In order to address whether expression of SOX1 could initiate neural differentiation and thereby substitute for the requirement of retinoic acid, we asked whether the transient exposure of P19 aggregates to retinoic acid can be replaced by a transient induction of RSVopSox1, through addition of IPTG. Wild-type P19 cells and transfected P19 clones (708-13, 708-16 and 708-21) were cultured as aggregates for 96 hours with or without the addition of IPTG. After 96 hours RNA was isolated from half of the aggregates for RNase protection and/or RT-PCR assays. The remaining aggregates were plated onto tissue culture substrate, allowed to differentiate for 3 days without further addition of IPTG and then scored for the expression of a panel of neuroepithelial and neuronal markers by immunocytochemistry. These conditions are the same as those used for retinoic acid-induced differentiation of wild-type P19 cells. As shown in Fig. 5C, after 96 hours the clones induced to express RSVopSox1 with IPTG expressed endogenous *Sox1* and *Mash1*. The expression of these two neuroepithelial markers was similar to that seen in wild-type cells induced with retinoic acid. In addition the IPTG induced clones expressed NESTIN (data not shown) and *Hoxa7* (Mahon et al., 1988) (Fig. 5D). Further differentiation of the transiently induced clones on substrate showed the presence of mature neurons as demonstrated by Neurofilament-positive (Fig. 7D), 3A10-positive (Fig. 7E) and Islet1-positive (Fig. 7F) cells. All three clones, 708-13 (data not shown), 708-16 (data shown) and 708-21 (data not shown) differentiated in this manner although the number of mature neurons produced was variable. The number of differentiated neurons formed in the IPTG induced clones was estimated by determining the number of Neurofilament-positve cells in a given field of cells (see Materials and Methods). The number of neurons range from 6-8% for clone 708-13, 15-20% for clone 708-16 and 20-25% for clone 708-21. The latter two clones show uniform and ubiquitous induction of SOX1 expression (see Fig. 4 for clone 708-16) whereas expression in clone 708-13 was not in all cells (data not shown). In addition, the transiently induced clones generated GFAP-positive cells indicating glial cell differentiation (data not shown). None of these markers was detected in wildtype P19 cells cultured in the presence of IPTG (not shown) or in clones 708-13, 708-16 (Fig. 7A-C), and 708-21 cultured in the absence of IPTG. As shown in Figs 1, 4 the expression of SOX1, both in vivo and in vitro, is mutually exclusive with mature neuronal markers such as Neurofilament and Islet1. To examine SOX1 expression in the mature neurons generated in the transiently induced clones, we used double-label immunofluorescence to simultaneously detect SOX1 and Neurofilament. No SOX1 expression could be detected in cells positive for Neurofilament in these cultures (Fig. 7G-I).

These results argue that transient induction of SOX1 bypasses the requirement for retinoic acid in promoting neural differentiation in P19 cells, and suggest that in vivo SOX1 may be involved in directing neural cell fate. In addition, these data support a close correlation between the down-regulation of SOX1 expression and neural differentiation.

DISCUSSION

In this paper we investigate the role of SOX1 in neural cell fate determination. We show that the expression of SOX1, both in vivo and in vitro, initiates at the time of neural induction and appears to be restricted to ectodermal cells committed to the neural fate. Expression of SOX1 is subsequently

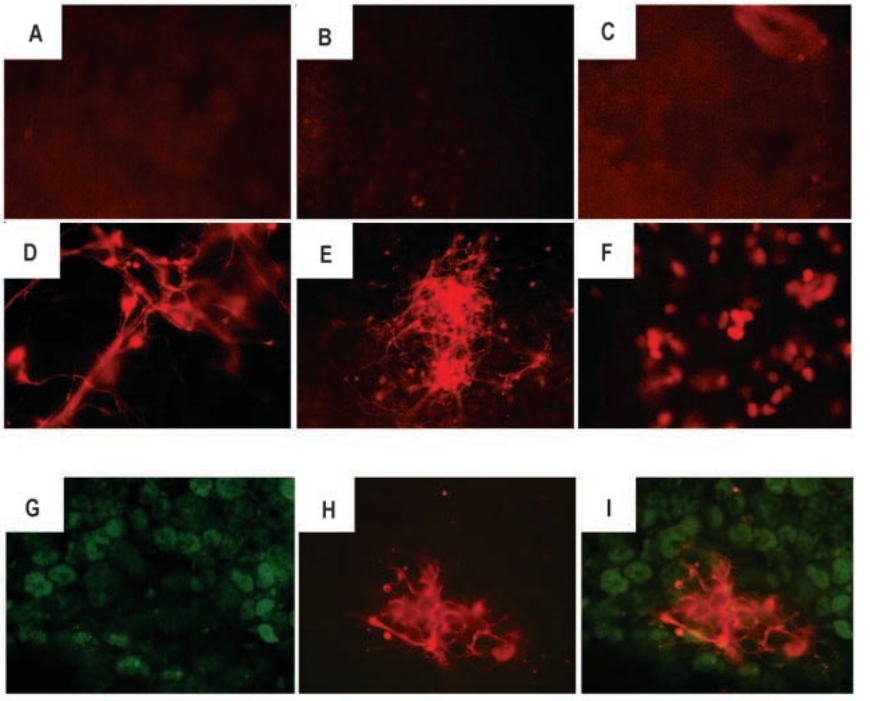

Fig. 7. Immunohistochemical analysis of neural differentiation induced by ectopic expression of SOX1 in P19-RSVopSox1 transformants. (A-C) 708-16 cells grown as aggregates for 96 hours, plated onto a substrate and after 4-5 days stained with anti-Neurofilament, anti-3A10, and anti-Islet1antibodies, respectively. No positive-staining cells were detected. (D-F) 708-16 cells grown as aggregates for 96 hours in the presence of IPTG, plated onto a substrate and after 4-5 days stained with anti-Neurofilament, anti-3A10, and anti-Islet1antibodies, respectively. Cells positive for all three markers were detected. (G-I) 708-16 cells grown as aggregates for 96 hours in the presence of IPTG, plated onto a substrate and after 4-5 days double labeled with anti-SOX1 and anti-Neurofilament antibodies. SOX1 and Neurofilament are mutually exclusive.

downregulated as neural cells exit mitosis to terminally differentiate. We demonstrate that induced expression of SOX1 in P19 embryonal carcinoma cells is able to promote neural differentiation and thereby substitute for the requirement of retinoic acid, thus providing evidence that the SOX1 transcription factor plays a role in biasing ectodermal cells to a neural fate.

SOX1: an early marker of neuroepithelial cells

The data reported here indicate that the onset of SOX1 expression is closely associated with the acquisition of neural fate by the ectoderm, both in vitro and in vivo. In vitro SOX1 expression is initiated within 24 hours of the addition of retinoic acid to P19 aggregates coincident with the induction of neuropithelial markers such as NESTIN, *Mash1* and *Wnt1*. In mouse and rat embryos SOX1 can first be detected in late primitive streak stage embryos and is restricted to cells of the antero/distal ectoderm. Previous fate mapping studies indicate that this region of the epiblast constitutes the primordium of the nervous system (Tam, 1989; Lawson et al., 1991; Quinlan et al., 1995; Tam and Zhou, 1996). Expression of SOX1 is detected throughout the cells of the neural plate and early neural tube along its entire anteroposterior axis. The early and uniform expression of SOX1 throughout the presumptive CNS indicates that SOX1 is activated by neural inducing signals and lends support to the proposal of a two step response of the ectoderm to organizer signals in generating a nervous system: neuralization followed by regionalization (Waddington and Needham, 1936; Storey et al., 1992; Streit et al., 1997). A similar uniform expression throughout the cells of the early neuroepithelium has been shown for two other members of the same *Sox* gene subfamily, *Sox2* and *Sox3* (Collignon et al., 1996; Collignon, 1992; Parsons, 1997). Moreover, expression of this *Sox* gene subfamily has been evolutionarily conserved. The *Drosophila* (Nambu and Nambu, 1996; Russell et al., 1996), zebrafish (Vriz et al., 1996) and avian (Uwanogho et al., 1995; Streit et al., 1997; Rex et al., 1997) putative orthologues of *Sox1*, *Sox2* and *Sox3* all show expression throughout the neural primordium. Thus, this subfamily of Sox genes represents a novel group of transcription factors which can serve as general early neuroepithelial markers.

A number of lines of evidence suggest that in the developing nervous system final commitment to a particular cell fate occurs late in the final progenitor cell division. In the cerebral cortex, it is well established that the generation of neurons results from a series of asymmetric divisions where one cell re-enters while the other exits the cell cycle (reviewed by McConnell, 1995). The multipotency of progenitors is lost as the cell progresses through the cell cycle. When transplanted early in their cell cycle (S phase), progenitor cells can take on the fate specified by the ectopic environment. However, when transplanted later (when the cell transits into the G_2 phase) the cells are committed to the fate of their origin (McConnell and Kaznowski, 1991). Similarly, the determination of motor neuron fate in the ventral horns of the spinal cord occurs in the final progenitor division (Ericson et al., 1996; Pfaff et al., 1996). The expression of SOX1 in the neural plate and neural tube seems to correlate with mitotically active progenitors that are not yet committed to a final fate. Both BrdU studies and antibody double labeling experiments provide evidence that the downregulation of SOX1 expression is coincident with neuroepithelial cells leaving the proliferative state throughout most of the CNS. In the embryonic spinal cord, SOX1 expression appears to be lost from both dorsal and ventral neural cells, which have undergone their final division and begun to terminally differentiate. However, this correlation has some exceptions in the anterior embryonic CNS: in the area of the optic vesicles, the downregulation of SOX1 appears to precede the exit of cells from mitosis (Collignon and Lovell-Badge, unpublished observation).

The timing of SOX1 expression as well as antibody double-labeling experiments presented here indicate that SOX1 is coexpressed with early markers of the onset of regional and positional differentiation and not with markers of terminal differentiation. The pattern of downregulation of SOX1 expression from ventrally located cells concurs with previous studies showing that floor plate differentiation precedes motor neuron differentiation. Our studies reveal, however, that two streams of neuroepithelial cells located between differentiated floor plate and motor neurons in 'region X', appear to be delayed in their commitment to terminal differentiation. This suggests the possibility that 'region X' cells, immediately adjacent to Sonic Hedgehog-expressing floor plate, may form a reservoir of precursor cells that ultimately differentiate into distinct ventral cell types.

SOX1 can impart neural fate to P19 cells

The induction of neural epithelium results in changes in the activity of transcription factors that regulate the expression of a large number of genes necessary to alter cell fate. The ability of the embryonal carcinoma cell line P19 to differentiate into neuronal cells in response to retinoic acid provides an in vitro assay which appears to closely resemble neural induction and differentiation (Bain et al., 1995). Thus, the differentiation of P19 cells in response to retinoic acid involves the ordered appearance and disappearance of genes that are observed in the newly induced and differentiating neural plate in vivo. Our results show that SOX1 can be included in the repertoire of genes expressed upon induction of neural tissue in vivo and in P19 cells. The onset of expression of SOX1 occurs within 24 hours after the addition of retinoic acid to P19 cells, coincident with the development of neuroepithelial precursors as defined by the expression of NESTIN, *Wnt1* and *Mash1*. The downregulation of SOX1 expression in vivo in postmitotic neurons is likewise mirrored in P19 cells.

The correlation between neural cell fate determination and onset of SOX1 expression led us to investigate if SOX1 alone can bias uncommitted P19 cells to a neural fate. Induction of endogenous *Sox1*, *Mash1* and *Hoxa7* was observed following transient ectopic SOX1 expression, indicating the presence of neuroepithelial precursor cells. The further differentiation of these induced cells into mature postmitotic neurons, expressing 3A10, Islet-1 and Neurofilament, as well as glial cells expressing GFAP, directly demonstrates that SOX1 can impart neural fate to P19 cells. The cascade of gene expression resulting from the misexpression of SOX1 in P19 cells was similar to that induced by retinoic acid.

A number of vertebrate transcription factors have previously been shown to impart neural fate on uncommitted ectodermal cells. Examples of these include XASH-3 (Ferriero et al., 1994; Turner and Weintraub, 1994) and CASH-4 (Henrique et al., 1997), ASH-3 (Takebayashi et al., 1997) *Xenopus* and chicken

homologues of the *Drosophila* AS-C genes; Neurogenin (Ma et al., 1996) and NeuroD (Lee et al., 1995), vertebrate homologues of the *Drosophila* atonal genes, and MyT1, a *Xenopus* C2HC zinc finger protein (Bellefoid et al., 1996). In animal cap assays, CASH-4, Neurogenin and NeuroD alone, as well as the combination of XASH-3 and MyT1, can induce neural differentiation in naive ectoderm in the absence of additional neural inducing molecules. This is analogous to the manner in which SOX1 promotes neural differentiation in P19 ectodermal cells in the absence of retinoic acid. However, the relatively late and restricted onset of expression of Neurogenin, NeuroD and MyT1, as well as the phenotype elicited by their misexpression in *Xenopus* embryos has led to the suggestion that these genes function in neuronal determination and differentiation rather than in neuroepithelial precursor determination. Only CASH-4, whose early expression in neural precursors is initiated by neural inducing signals and whose ectopic expression leads to the expansion of the neural plate, seems to function to promote the formation of neuroepithelial precursors. However, in vivo, the expression of CASH-4 is restricted to the posterior neural plate and therefore may play a role in specifying just posterior neural fate. The early expression of SOX1 throughout the anteroposterior axis of the neural plate and its ability to elicit a neural response in P19 cells implicates a general role for SOX1 in neuroepithelial cell fate determination.

SOX genes and neural determination in vivo

Our data show that SOX1 expression is intimately associated with the acquisition of neural fate in vivo and in vitro and that SOX1 expression can by itself induce neural fate in uncommitted P19 cells. An as yet unanswered question is whether misexpression of SOX1 is also sufficient to elicit a similar response in vivo. It is also essential to establish the degree to which SOX1 is required for neuroepithelial differentiation. It is likely, in fact, that there is functional redundancy with other members of the *Sox* gene family. The members of the subfamily of *Sox* genes, which includes *Sox1*, are closely related within the HMG box (approximately 90% homology) (Gubbay et al., 1992; Denny et al., 1992) and show significant homology to one another outside the HMG box in both N-terminal and C-terminal domains. In particular, SOX2 and SOX3 have very similar properties to SOX1 and all three are coexpressed throughout the early CNS (Collignon et al., 1996). Moreover, the three proteins can substitute for one another in cotransfection assays. (Kamachi et al., 1995; Y. Kamachi, M. Uchikawa, J. Collignon, R. L.-B. and H. Kondoh, unpublished data). Studies of mice carrying targeted mutations in *Sox1* and *Sox2* further support the notion of functional redundancy: gross morphological effects are only apparent in sites where the genes are expressed uniquely. *Sox2* homozygous null embryos die just after implantation, reflecting the unique expression of *Sox2* in the inner cell mass and epiblast (S. Nicolis, A. Avilion, L. H. P., L. Perez, N. Vivian and R. L.-B., unpublished data). Mice carrying a homozygous mutation of *Sox1* display abnormalities in lens formation, where neither *Sox2* nor *Sox3* are expressed but show only mild defects within the CNS (Nishiguchi et al., 1998) where both *Sox2* and *Sox3* are normally expressed.

The early expression of SOX1 throughout the neural plate and neural tube, the correlation between its expression and proliferating neural epithelial cells and its subsequent downregulation after the exit of neural cell from mitosis implicates it as a marker of neural progenitor cells in vivo. Moreover, our studies on induction of SOX1 expression in P19 cells suggest it may be at least partially responsible for the acquisition of neural cell fate. Further experiments will determine the extent to which this subfamily of *Sox* genes operates in vivo to define neural tissue.

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