Development 127, 2367-2382 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV1521

Sox2 regulatory sequences direct expression of a β-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells

Massimo V. Zappone[1,§], Rossella Galli[3,§], Raffaella Catena[1,*,§], Natalia Meani[1], Silvia De Biasi[4], Elisabetta Mattei[5,6], Cecilia Tiveron[5], Angelo L. Vescovi[3], Robin Lovell-Badge[7], Sergio Ottolenghi[1,*] and Silvia K. Nicolis[1,2,*,‡]

[1]Dipartimento di Genetica e di Biologia dei Microrganismi, Università di Milano, via L.Celoria 26, 20133 Milano, Italy
[2]Dipartimento di Biologia Strutturale e Funzionale, Universita' dell'Insubria, via Dunant 3, 21100 Varese, Italy
[3]Istituto Neurologico 'C. Besta', via Celoria 11, 20133 Milano, Italy
[4]Dipartimento di Fisiologia e Biochimica generali, Università di Milano, via L.Celoria 26, 20133 Milano, Italy
[5]Istituto Regina Elena, via delle Messi d'oro 156, 00158 Roma, Italy
[6]Istituto di Tecnologie Biomediche del CNR, v.le Marx 43, 00143 Roma, Italy
[7]MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW71AA, UK
*Present address: Dipartimento di Biotecnologie e Bioscienze, Universita' di Milano – Bicocca, p.zza della Scienza 2, 20126 Milano, Italy
§Joint first authors
‡Author for correspondence at present address (e-mail: silvian@mailserver.unimi.it)

Accepted 9 March; published on WWW 10 May 2000

SUMMARY

***Sox2* is one of the earliest known transcription factors expressed in the developing neural tube. Although it is expressed throughout the early neuroepithelium, we show that its later expression must depend on the activity of more than one regionally restricted enhancer element. Thus, by using transgenic assays and by homologous recombination-mediated deletion, we identify a region upstream of *Sox2* (–5.7 to –3.3 kb) which can not only drive expression of a *β-geo* transgene to the developing dorsal telencephalon, but which is required to do so in the context of the endogenous gene. The critical enhancer can be further delimited to an 800 bp fragment of DNA surrounding a nuclease hypersensitive site within this region, as this is sufficient to confer telencephalic expression to a 3.3 kb fragment including the *Sox2* promoter, which is otherwise inactive in the CNS.**

Expression of the 5.7 kb *Sox2β-geo* transgene localizes to the neural plate and later to the telencephalic ventricular zone. We show, by in vitro clonogenic assays, that transgene-expressing (and thus G418-resistant) ventricular zone cells include cells displaying functional properties of stem cells, i.e. self-renewal and multipotentiality. We further show that the majority of telencephalic stem cells express the transgene, and this expression is largely maintained over two months in culture (more than 40 cell divisions) in the absence of G418 selective pressure. In contrast, stem cells grown in parallel from the spinal cord never express the transgene, and die in G418. Expression of endogenous telencephalic genes was similarly observed in long-term cultures derived from the dorsal telencephalon, but not in spinal cord-derived cultures.

Thus, neural stem cells of the midgestation embryo are endowed with region-specific gene expression (at least with respect to some networks of transcription factors, such as that driving telencephalic expression of the *Sox2* transgene), which can be inherited through multiple divisions outside the embryonic environment.

Key words: *Sox* genes, Neural stem cells, CNS patterning, Neural development, Gene regulation

INTRODUCTION

Regional specification in the developing central nervous system (CNS) is related to the establishment of profiles of differential gene expression. Two aspects can be distinguished in this process. The first one is patterning. All of the cells of the CNS arise from the neuroepithelium, the initially morphologically uniform layer of cells that folds to form the neural tube; patterning of the neuroepithelium leads to its progressive subdivision into regions with distinct fates (Lumsden and Krumlauf, 1996; Beddington and Robertson, 1998; Rubenstein et al., 1998; Ruiz i Altaba, 1998).

The second aspect is cell differentiation. The proliferating, undifferentiated neuroepithelium will give rise to postmitotic, differentiated cell types that constitute the mature CNS, i.e. neurons, astrocytes and oligodendrocytes. Within these three major cell categories, various specialized cell types exist, which are specific to individual regions of the CNS, and

confer on them their specific functional characteristics (Jacobson, 1991).

Neuroepithelial stem cells have been characterized throughout the developing CNS (McKay, 1997; Stemple and Mahanthappa, 1997) including the telencephalon. These cells are able to maintain a germinal population by self-renewal, as well as to generate differentiated progeny of all three main CNS cell types. The genetic regulatory mechanisms active in these cells, and in their immediate progeny, that allow them to give rise to differentiated cells specific to the different CNS functional regions, are largely unknown. In particular, we need to understand how regional specification mechanisms integrate with stem cell differentiation mechanisms to produce region-specific gene expression and differentiation (Stemple and Mahanthappa, 1997; Morrison et al., 1997).

The *Sox2* gene encodes a highly conserved transcription factor (Collignon et al., 1992, 1996; Stevanovic et al., 1994; Uwanogho et al., 1995; Mizuseki et al., 1998; Uchikawa et al., 1999). Mouse *Sox2* is first expressed in the totipotent cell lineage, in particular in the blastocyst inner cell mass and in the embryonic ectoderm, and in germ cells. Targeted mutagenesis has shown that it is essential for primitive ectoderm survival (S. K. N., A. Avilion, L. Pevny, R. L.-B., unpublished data). After gastrulation, *Sox2* is expressed in the neural tube from the earliest stages of its development (neural plate) (Collignon, 1992; Uwanogho et al., 1995; Uchikawa et al., 1999; Wood and Episkopou, 1999). In *Xenopus*, *Sox2*, acting in conjunction with bFGF, was shown to have neuralizing activity (Mizuseki et al., 1998).

We have explored the regulation of *Sox2*, by transgenic experiments, and identified flanking regulatory regions that drive region-specific transgene expression to the developing telencephalon, predominantly in its dorsal aspect; in addition, in this context, expression is directed to the undifferentiated precursor/progenitor cells of the ventricular zone. The identified sequences are essential for dorsal telencephalic expression of the endogenous *Sox2* gene, as shown by homologous recombination-mediated deletion.

Further, we have investigated the expression of the *Sox2* transgene in stem cell-enriched cultures derived from the dorsal telencephalon, as well as from the spinal cord region of transgenic embryos. Through clonogenic assays, we show that the *Sox2* transgene is expressed in cells with functional stem cell properties, and find that the regional specificity of expression observed in vivo is present already at the neural stem cell level, and is maintained throughout extended in vitro culture.

MATERIALS AND METHODS

DNA constructs

To generate the constructs shown in Table 1, fragments containing different extensions of Sox2 5′ flanking sequences were obtained by cutting cloned DNA (from a 129 genomic library in λFIXII) at a common *Not*I site (in the 5′ untranslated *Sox2* region) and at additional restriction enzyme sites, located at various positions further upstream in the 5′ flanking region. These fragments were then ligated into the *Not*I site upstream of the *β-geo* gene, in the pSAβ-geobpA vector (Friedrich and Soriano, 1991), from which the splice acceptor (SA) site had been deleted (by *Spe*I and *Hin*dIII digestion, blunting and religation).

In particular, the 12 kb 5′ flanking sequence was obtained by *Not*I digestion, exploiting an upstream *Not*I site which is part of the λFIXII polylinker, into which the 129 DNA had been cloned (from now on denoted as the ‘polylinker *Not*I site’). The 5.7 kb 5′ flanking sequence was derived by similarly exploiting the ‘polylinker *Not*I site’ from a different genomic clone, harboring a shorter Sox2 5′ flanking region. In the 12+2.5 kb *Sox2* construct, a further 2.5 kb *Sal*I fragment (derived from a genomic region about 1 kb downstream of the *Sox2* gene) was ligated into a unique *Sal*I site 3′ to *β-geo*.

To generate *Sox2β-geo* fragments for transfections and injections (12 kb, 5.7 kb), we exploited a polylinker-derived *Sal*I site that lies immediately 3′ to the ‘polylinker *Not*I site’; as a *Sal*I site lies 3′ to the *β-geo* poly(A) addition site, *Sal*I digestion releases the desired *Sox2β-geo* fragment. The 3.3 kb *Sox2β-geo* fragment was released by digestion of the 5.7 kb *Sox2β-geo* construct with *Hin*dIII (in the 5′ flanking region) and *Sal*I. The 12+2.5 kb *Sox2β-geo* construct was obtained as a *Kpn*I linearized molecule.

To generate the HSI 3.3 kb construct (Table 1), the 800 nt region surrounding HSI was amplified by PCR using the following primers: 5′ TTA CGT ACA AGC TTG TCG ACG TCA AAT AGG GCC CTT TTC AG 3′ (upstream primer, containing a *Hin*dIII site added for cloning, and a *Sal*I site added for subsequent transgene excision).

5′ TGC ATT CGA AGC TTA AGC CAA CTG ACA ATG TTG TGG 3′ (downstream primer, containing a *Hin*dIII site added for cloning). The fragment was cloned in the ‘sense’ orientation into the 5′ *Hin*dIII site of the 5.7 kb construct, and released for injection by *Sal*I digestion.

To generate the targeting vector (Fig. 2A), a 5.5 kb fragment spanning the most 5′ portion of the *Sox2* 5′ flanking region (region A in Fig. 2A) was obtained by digestion at the upstream ‘polylinker *Not*I site’ (see above) and at a downstream *Xho*I site; this was connected to a *Hin*dIII-*Sal*I fragment, comprising the proximal *Sox2* 3.3 kb 5′ flanking region (region B in Fig. 2A) and *β-geo* (isolated from the 5.7 kb *Sox2* construct); the junction was via an adaptor converting the *Hin*dIII site into a *Sal*I site. This creates a deletion including the −5.7 to −3.3 kb region within an intermediate plasmid which harbors the 5′ region of homology of the targeting construct. From this, a *Not*I fragment (spanning the ‘polylinker *Not*I site’ and the *Not*I site upstream to *β-geo*), carrying the ‘deleted’ *Sox2* 5′ flanking sequences, was isolated and used to replace the corresponding non-deleted fragment in the 12+2.5 kb *Sox2β-geo* construct (carrying also the 3′ region of homology). The construct was linearized with *Kpn*I (just 3′ to the downstream *Sal*I site) prior to electroporation.

ES cell transfection

CCE ES cells were electroporated at 2×10^7 cells/0.65 ml PBSCM (PBS+1 mM $CaCl_2$, 1 mM $MgCl_2$) with a BioRad Gene Pulser at 220 V, 960 μF. After 5 minutes on ice, cells were plated on three 10 cm dishes onto STO feeder layers (Hogan et al., 1994). G418 selection at 300 μg/ml was applied after 36-48 hours; resistant clones were picked at day 9-11 and expanded for freezing, DNA analysis and blastocyst injection.

Generation of transgenic mice through blastocyst and pronuclear injection

ES cells from stably transfected or targeted clones were microinjected into blastocysts from crosses between C57BL/6xDBA females and C57BL/6 or C57BL/6xDBA males, as described by Hogan et al. (1994). ES cell contribution to the embryos was evaluated through multiplex PCR on their DNA. A primer pair for the transgene, and one for the endogenous *Sox2* gene as an internal reference were used; the ratio between the transgenic band and the endogenous band was evaluated, in the linear range of the PCR reaction. A PCR on DNA of the ES cell clone used represented ‘100%’ chimaerism; a scale of known dilutions of this into wild-type DNA was generated, and the percentage of chimaerism of the embryo was evaluated with this scale as a reference. Primers were: for the transgene, 5′ACAGTCCTGGCCGGGCGGAG3′ (in the *Sox2* 5′UTR) and 5′AGATGGGCGCATCGTAACCGTG3′ (in

β-geo); for *Sox2*, 5′GGCAGCTACAGCATGATGCAGGAGC3′ and 5′CTGGTCATGGAGTTGTACTGCAGG3′.

Zygotes for pronuclear DNA injection were from crosses between superovulated C57BL/6xDBA females and C57BL/6xDBA males. Procedures were as described by Hogan et al. (1994). Lines were propagated through breeding with B6D2F1 animals.

Assay of β-galactosidase activity and immunohistochemistry on transgenic embryos

Embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP40 in PBS for 30-90 minutes at 4°C. After several washes in 0.02% NP40 in PBS, they were stained at 30°C in the dark in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.02% NP40, 1 mg/ml X-gal in PBS. Adults were perfused in 30% sucrose in PBS, the brain was removed, embedded in OCT, and sectioned with a cryostat. Sections were post-fixed for 30 minutes in 1% glutaraldehyde in PBS, washed and stained for β-gal activity as above.

Embryos were post-fixed in 4% paraformaldehyde in PBS for 12-24 hours, embedded in 6% agarose and sectioned in the coronal plane at 80 μm intervals on a vibratome. Free-floating sections were collected in PBS.

7.5 d.p.c. embryos were osmicated, embedded in Epon-Spurr and cut in 2 μm semithin sections. Selected X-gal-stained brain sections were subsequently processed for immunoistochemistry to detect calretinin, a calcium-binding protein expressed in specific subpopulations of neurons and appearing early during the fetal period in rodents (Fonseca et al., 1995), using a polyclonal antiserum (Swant, Bellinzona, Switzerland, 1:2500). Sections were incubated overnight with the primary antibody, followed by the biotinylated secondary antibodies (goat anti-mouse, Vector, Burlingame, CA) for 1 hour, and by the avidin-biotin peroxidase complex (ABC, Vector) for 1 hour. The reaction was visualized using diaminobenzidine and H_2O_2 and the immunoreacted sections were mounted on gelatinized slides with Permount.

Nuclease hypersensitivity experiments

Nuclei were prepared from exponentially growing cells in RSB (10 mM Tris/HCl, pH 7.5, 10 mM NaCl and 3 mM $MgCl_2$) containing 0.1%(v/v) NP40 according to the procedure of Forrester et al. (1990), and resuspended in RSB. Aliquots were incubated for 5 minutes at 37°C either without or with DNAse I (Boehringer) over a range of 0.2-10 μg/ml. Genomic DNA was isolated, digested with appropriate enzymes and analyzed by Southern blotting.

For *Pst*I sensitivity, nuclei were prepared as published previously (Bresnik et al., 1994). Washed nuclei were resuspended in 1 ml of digestion buffer (10 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 0.1 mg/ml BSA), divided into aliquots and incubated without enzyme or with 50 or 100 units of *Pst*I for 30 min at 37°C. Genomic DNA was extracted, digested with *Hin*dIII and analyzed by Southern blotting.

Culture of neural stem cells from brain and spinal cord of transgenic embryos

Brains from 14.5 d.p.c. embryos were dissected, and the two cerebral hemispheres were separated; one of the hemispheres was tested for β-gal activity to identify transgenic embryos, the other one was used for cell isolation. The cortex was removed and triturated using a fire-polished Pasteur pipette. In parallel, spinal cords from the same transgenic embryos were isolated and processed in the same way. Cell suspensions were plated onto untreated 12-well tissue culture plates (Nunc, USA), in the presence of, respectively, 20 ng/ml and 10 ng/ml of human recombinant EGF and FGF2 in NS-A basal serum-free medium (a DMEM-F12 medium whose nutrient composition is optimized for neural stem cell growth; Euroclone, Irvine, Scotland known as ‘stem cell medium’) containing 2 mM L-glutamine, 0.6% glucose, 9.6 g/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin (sodium salt, grade II, Sigma). Under these conditions, cells rapidly grow to form neurospheres (Gritti et al., 1996). After 4 days, cultures were harvested, mechanically dissociated and replated under the same conditions. After performing this procedure twice to eliminate short term dividing precursors, bulk cultures were generated by passaging cells at higher density (10^4 cells/cm^2) every 3-4 days in the same growth medium. Cell counting and viability tests were performed at every passage by Trypan Blue exclusion. When required, G418 was added at a concentration of 200 μg/ml.

Cloning and serial subcloning procedure

Neurospheres at the third passage in G418 were dissociated into single cells, which were plated at clonal density (less than 1 cell/cm^2) in the medium described above (with G418) in methylcellulose (1.5% final concentration; Dow Methocell) in 35 mm Petri dishes; about 15% of plated cells proliferated to give rise to secondary clonal spheres by 10-15 days after plating (DAP).

Secondary individual clones were either differentiated, or assayed for the ability to generate tertiary spheres. For differentiation, spheres were transferred onto Matrigel™-coated glass coverslips (1 sphere/coverslip), grown for 3 days in stem cell medium (see above) with FGF2, left 5 days in ‘control medium’ (i.e. stem cell medium without EGF and FGF2, and supplemented with 1% foetal calf serum), and processed for triple antigen indirect immunofluorescence. Alternatively, to assay for self-renewal, spheres were transferred into Eppendorf 2 ml tubes (1 sphere/tube), mechanically dissociated to a single cell suspension and plated onto 24-well plates for subcloning experiments (in stem cell medium with G418). 30% of the viable cells proliferated and gave rise to tertiary spheres whose number in each dish was assessed at 12 DAP. Some of the tertiary spheres were plated onto glass coverslips (1 sphere/coverslip) and processed for triple antigen immunofluorescence assay. The rest underwent a second subcloning step.

Immunocytochemistry

Single spheres grown on Matrigel™-coated glass coverslips were fixed (20 minutes) with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and rinsed 3 times with PBS. Coverslips were then incubated for 90 minutes at 37°C in PBS containing 10% normal goat serum (NGS), 0.3% Triton X-100 and the appropriate primary antibodies or antisera. Following thorough washing with PBS/10% NGS, cells were reacted for 45 minutes (room temperature) with secondary fluorescein (FITC)- or rhodamine (TRITC)-conjugated goat anti-mouse or anti-rabbit IgG antibodies (1:100, Boehringer Mannheim) or with donkey anti-mouse IgM antibodies coupled to 7-amino-4-methylcoumarin-3-acetic acid (AMCA; 1:100, Jackson). Coverslips were rinsed 3 times in PBS, once in distilled water and mounted on glass slides with Fluorsave (Calbiochem). Primary antibodies/antisera used were: mouse monoclonal anti-microtubule-associated protein-2 (MAP-2, IgG, 1:200, Boehringer), anti-tau-microtubule associated protein (Tau-1; IgG, 1:200, Boehringer), anti-O4 (IgM; 1:200, Boehringer) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:5, Incstar).

Evaluation of the stability of transgene expression in long-term cultures of neural stem cells by a clonogenic assay

Neural stem cells obtained both from the cortex and the spinal cord of 14 d.p.c. transgenic embryos were expanded in the absence of G418 under the conditions described above for up to 3 months. At successive intervals, starting from day 0, aliquots of cells were collected and plated onto polyornithine-treated 48-well plates at a density of 2000 cell/cm^2; half were cultured in the absence, and half in the presence of G418 (200 μg/ml). For each time point, six or more independent wells were set up (with and without G418). After 5-7 days in vitro (DIV), the number of clones generated under each condition was assessed and the percentage of G418-resistant clones evaluated.

RT-PCR analysis of transgenic neurospheres

Total RNA was extracted from neurospheres or embryonic tissue by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). 1-2 µg of RNA were reverse transcribed for 1 hour at 37°C with Superscript reverse transcriptase (GIBCO-Life Technologies), using random hexamers (200 ng/reaction) as primers in a total volume of 20 µl, according to the supplier's instructions. To compensate for slightly variable RNA and cDNA yields, the amount of cDNA synthesized was calibrated by using the relative expression level of the housekeeping gene HPRT as a standard. Amounts of cDNA yielding equivalent amounts of HPRT amplification product (between 1/30 and 1/70 of the RT reaction) were used in subsequent PCR reactions.

cDNAs were amplified using the following primers:

HPRT: 5′ CCT GCT GGA TTA CAT TAA AGC ACT G 3′

5′ GTC AAG GGC ATA TCC AAC AAC AAA C 3′ (annealing: 53°C; 352 bp band on cDNA)

SOX2: 5′ GGC AGC TAC AGC ATG ATG CAG GAG C 3′

5′ CTG GTC ATG GAG TTG TAC TGC AGG 3′ (annealing: 67°C; 130 bp band on cDNA)

BF-1: 5′ GGG CAA CAA CCA CTC CTT CTC CAC 3′

5′ GAC CCC TGA TTT TGA TGT GTG AAA 3′ (annealing: 60°C; 396 bp band on cDNA)

OTX-1: 5′ ACC ACC TTC ACG CGC TCA CA 3′

5′ CAC CTC CTC GCG CAT GAA GA 3′ (annealing: 60°C; 87 bp band on cDNA)

TBR-2: 5′-CACGTCTACCTGTGCAACCG-3′

5′-CCTGTCATTTTCTGAAGCCGT-3′ (annealing: 60°C; 620 bp band on cDNA)

EMX-2: 5′ GTC CCA GCT TTT AAG GCT AGA 3′

5′ CTT TTG CCT TTT GAA TTT CGT TC 3′ (annealing 60°C; 151 bp band on cDNA).

PCRs were performed in 50 µl in standard conditions (15 mM $MgCl_2$, dNTP 2 mM each, 1 U Taq polymerase).

RESULTS

Sox2 flanking sequences direct expression of a *β-geo* transgene to the developing telencephalon

In an initial search for sequences regulating *Sox2* expression during embryogenesis and nervous system development, several constructs were generated in which DNA sequences flanking the *Sox2* coding region were fused to a *β-geo* reporter gene (Friedrich and Soriano, 1991), which encodes for β-galactosidase activity (*lacZ*) as well as G418 resistance (Table 1).

These constructs were used to generate transgenic mouse embryos, either by pronuclear injection, or by ES cell transfection followed by generation of chimaeras by blastocyst injection. As *Sox2* is expressed in ES cells, the latter procedure has the advantage, relative to conventional transgenic practice, that integrations in chromatin regions permissive for transgene expression are likely to be selected, versus those in non-permissive regions; in addition, the copy number is usually low (Bradley and Liu, 1996).

Of note, all the constructs had significant activity in ES cells, as compared to the promoterless control (data not shown); all ES clones used for chimaera experiments showed comparable *lacZ* expression in culture.

When assayed at mid-gestation (12.5 d.p.c.), the 12+2.5 kb *Sox2*, 12 kb *Sox2*, and 5.7 kb *Sox2* constructs all gave strong *lacZ* expression confined to the developing telencephalon (Fig. 1B; Table 1). This is a subset of the *Sox2*-expressing domains in the CNS, which can be illustrated by comparing it with the expression of a *β-geo* gene inserted into the *Sox2* locus by homologous recombination (i.e. $Sox2^{\beta\text{-}geo}$; see Fig. 1A). In contrast, the 3.3 kb *Sox2* transgene failed to give any detectable telencephalic activity. Ectopic expression was instead observed (Fig. 1C), or no detectable expression at all (see Table 1). This indicated that the 5.7 kb *Sox2* construct contains regulatory elements critical for this region-specific telencephalic expression, which are missing in the 3.3 kb *Sox2* construct.

Low levels of expression were observed, with individual integrations of the 12+2.5 kb, 12 kb and 5.7 kb *Sox2β-geo* constructs, in other sites such as retina, olfactory epithelium, diencephalon, some PNS ganglia (data not shown), which also belong to the endogenous *Sox2*-expressing tissues. This expression may reflect the action of regulatory sequences more vulnerable to position effects, or more dependent on the context of the endogenous gene for their activity.

Dorsal telencephalic expression of the endogenous *Sox2* gene requires the –5.7/–3.3 kb region

The results shown above indicate that *Sox2* regulatory sequences can be isolated, which direct transgene expression only to a specific subset of the normal (i.e. panneural) *Sox2* expression domain.

How important are these sequences within the intact endogenous gene? To address this question, we removed the putative critical sequences (i.e. the region between −5.7 kb and −3.3 kb) from one of the two copies of the endogenous *Sox2* gene by homologous recombination in ES cells, and we subsequently assayed for expression in chimaeric embryos. The targeting vector for this experiment (Fig. 2A) carries a deletion in the 5′ homology region relative to the wild-type locus, corresponding to the sequences critical for telencephalic

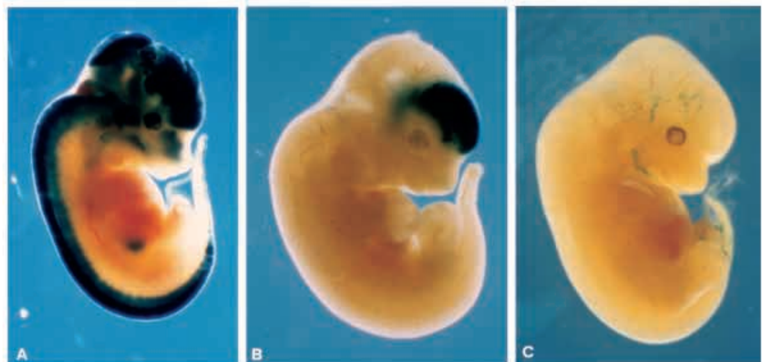


Fig. 1. β-gal activity in *Sox2β-geo* transgenic embryos at midgestation. (A) 12.5 d.p.c. embryo heterozygous for a *β-geo* gene inserted at the *Sox2* locus by homologous recombination ($Sox2^{\beta\text{-}geo}$), replacing the *Sox2* protein coding region (as in allele 2, Fig. 2A). (B,C) Chimaeric 12.5 d.p.c. embryos generated with ES cell clones stably transfected with the 5.7 kb *Sox2* (B) or the 3.3 kb *Sox2* (C) construct.

Table 1. Sox2 β-geo constructs and expression in 12.5 d.p.c. transgenic embryos

Construct	Number of transgene integrations tested	β-gal expression		
		Telencephalon	Ectopic	None
12+2.5 kb	3*	3	0	
12 kb	3*	3	0	
5.7 kb	12 (3*+4‡+5§)	7 (2*+2‡+3§)	2 (1*+1§)	3 (2‡+1§)
3.3 kb	9 (5*+4‡)	0	3*,¶	6 (2*+4‡)
HS 1+3.3 kb	9‡	5‡	3‡,**	1

*Stably transfected ES cell clones used to generate chimaeric embryos (30-90% chimaerism).
‡Transient transgenic embryos generated by pronuclear injection.
§Transgenic lines.
¶Sites of ectopic expression were surface ectoderm or abdominal region.
**A weak telencephalic expression was observed, together with ectopic expression in surface ectoderm and/or hind limbs.
In the diagram, continuous lines indicate the genomic sequences immediately upstream and downstream (for the 12 + 2.5 kb construct) of the single *Sox2* coding exon (see Materials and Methods). The black box indicates the *β-geo* reporter gene to which they were fused. The vertical arrow indicates the nuclease hypersensitive site 1 (see Results).

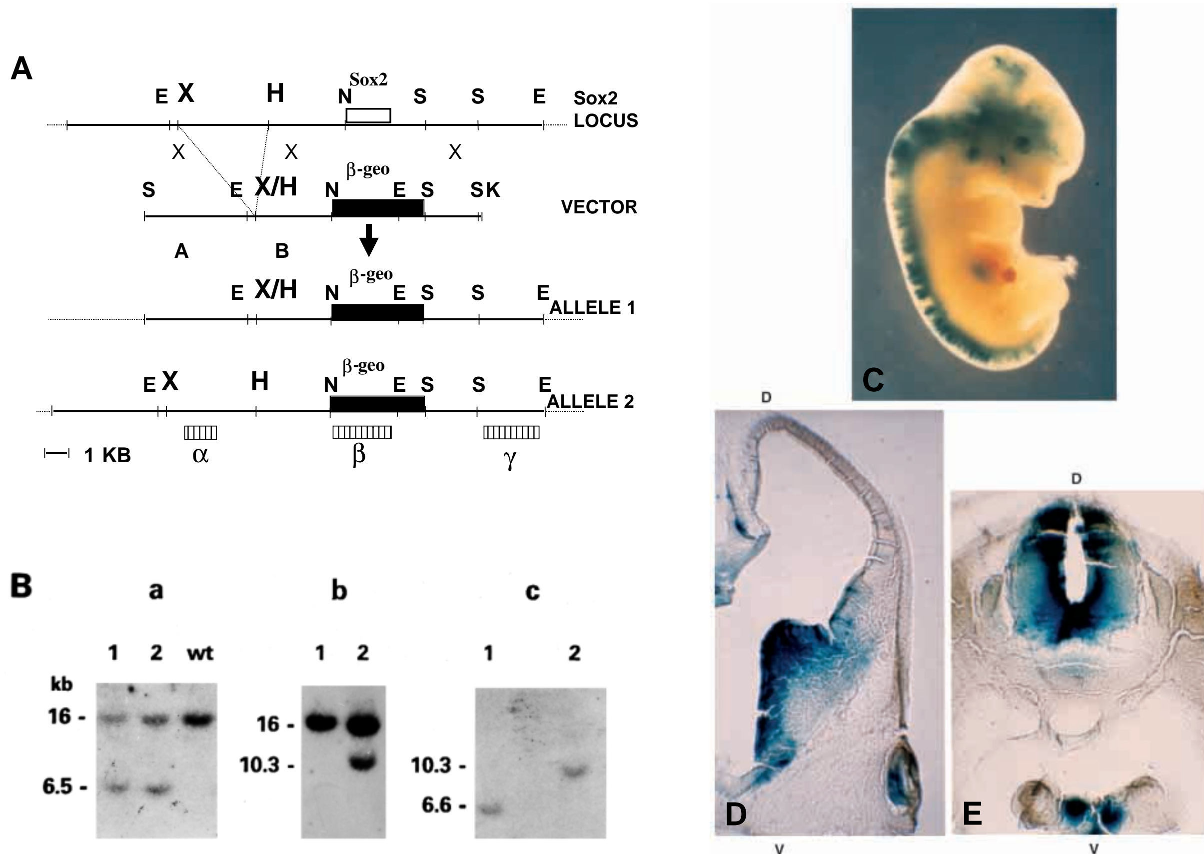


Fig. 2. Targeted deletion of regulatory elements from the *Sox2* locus. (A) Gene targeting vector and alleles generated by homologous recombination. Allele 1 (with 5′ deletion, $Sox2^{\beta\text{-}geo\Delta E}$) and allele 2 (with wild-type 5′ flanking sequences, $Sox2^{\beta\text{-}geo}$) result from recombination in region A or region B, respectively. (B) Southern analysis of ES cell clones. DNA was digested with *Eco*RI and hybridized with different probes. (a) Probe γ (a *Sal*I-*Eco*RI fragment), to identify clones where homologous recombination has occurred on one of the two chromosomes, resulting in a 6.5 kb band (lanes 1 and 2). The wild-type allele gives a band of approximately 16 kb. (b,c) Probes α and β to distinguish between alleles 1 and 2. (b) Probe α does not hybridize to allele 1 (lane 1, 16 kb band originating from the wild-type allele), but hybridizes to a 10.3 kb fragment in allele 2 (lane 2). (c) Probe β, hybridizes to a 6.6 fragment in allele 1 (lane 1), and to a 10.3 fragment in allele 2 (lane 2). E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; S, *Sal*I; X, *Xho*I; X/H, the fusion between the *Xho*I and *Hin*dIII sites. (C) Chimaeric 12.5 d.p.c. embryo generated with ES cells carrying allele 1 ($Sox2^{\beta\text{-}geo\Delta E}$, i.e. flanking sequences with deletion). (D,E) Transverse sections through the telencephalon (D) and spinal cord (E) of the chimaera shown in C. D, dorsal; V, ventral. Besides the neuroepithelium, the X-gal staining is also visible in the retina (see D) and gut (see E), which also belong to endogenous *Sox2*-expressing domains (Collignon 1992; our unpublished observations).

transgene expression. In addition, the *Sox2* coding sequences are substituted by the *β-geo* reporter gene. *β-geo* is thus driven by the *Sox2* flanking sequences; this will allow it to act as a reporter gene, as well as a selectable marker in ES cells (which express *Sox2*). Upon homologous recombination, two different alleles can be generated (see Fig. 2A). Following recombination in region B, the *Sox2* coding region is substituted with *β-geo*, but the 5′ flanking regions are left unaltered (Fig. 2A, allele 2). Allele 2 is identical to the previously generated *Sox2* knock-out allele $Sox2^{\beta\text{-}geo}$ (see the expression pattern in heterozygous embryos in Fig. 1A) (S. K. N., A. Avilion, L. Pevny, R. L.-B.,unpublished data). β-gal expression from this allele is expected to mirror the endogenous *Sox2* expression pattern (Fig. 1A). If, however, homologous recombination takes place in region A, *β-geo* is equally substituted in place of *Sox2*, but the 5′ flanking sequences, that drive its expression, carry the deletion (Fig. 2A, allele1, i.e. $Sox2^{\beta\text{-}geo\Delta E}$). The consequences of the deletion of the regulatory sequences on the linked *β-geo* reporter can thus be studied.

Following transfection in embryonic stem cells and G 418 selection, 14 homologous recombinant clones were isolated and further characterized. Southern analysis (Fig. 2B) showed that 6 of these clones contained the allele with the wild-type 5′ flanking sequences (allele 2), whereas 8 contained the allele with the 5′ deletion (allele 1). All these clones also stained positive for β-galactosidase activity; the intensity of staining varied slightly between clones, but with no apparent correlation with the presence of one allele rather than the other. In ES cells, therefore, the targeted deletion did not make any appreciable difference (with respect to wild-type sequences) in the ability to give rise to neomycin-resistant clones, or to produce β-galactosidase activity.

Mid-gestation chimaeric embryos harboring an allele with wild-type 5′ flanking sequences (allele 2), exhibit (as expected) a panneural β-gal staining profile (not shown) that is essentially identical to that observed (see Fig. 1A) in previously generated embryos heterozygous for allele 2, i.e.$Sox2^{\beta\text{-}geo}$. In particular, different regions of the CNS, such as the forebrain, hindbrain and spinal cord all show an intense, very similar degree of staining; homogeneity of expression levels can be appreciated also by the simultaneous appearance of a visible X-gal precipitate in all these structures, including the telencephalon, notably its dorsal part, after short incubation periods. When clone 7.2, harboring the 5′ deletion (Fig. 2A, allele 1, i.e. $Sox2^{\beta\text{-}geo\Delta E}$), was used to generate chimaeric embryos, β-galactosidase staining in the posterior CNS regions, such as the spinal cord and the hindbrain, appeared readily (in about 2 hours, as with the non-deletion allele). In contrast, β-gal expression in the forebrain, and particularly in the telencephalon, appeared severely disturbed. In particular, within the telencephalon, the staining appeared to be mainly in the ventral part, whereas in the dorsal part the staining was very low, even upon prolonged incubation, as compared to posterior regions (Fig. 2C). Indeed, sectioning (Fig. 2D,E) confirmed the nearly complete absence of expression in the dorsal telencephalon (notably the site of strongest expression of the 5.7 kb transgene, see below, Fig. 4) indicating that the visible staining was due to strong β-gal activity in the ventral part, where the ganglionic eminences are developing (Fig. 2D). Here, the staining is comparable to that observed in the spinal cord (Fig. 2E). In addition to the strong effect on the dorsal telencephalon, there is some decrease in β-gal staining in mesencephalon, diencephalon, and some ganglia, that may not have been expected on the basis of the transgenic data. As this analysis was carried out on chimaeric embryos, an evaluation of absolute expression levels is not possible. However, the internal comparison between different CNS regions shows that the deletion of the −5.7 to −3.3 kb region from the *Sox2* locus acts on gene expression in a selective fashion, affecting the dorsal telencephalon much more severely than the ventral telencephalon (and more posterior CNS regions). Furthermore, the deletion has comparatively little, if any, influence on expression in ES cells.

We conclude that the −5.7 to −3.3 kb region is essential for proper in vivo regulation of *Sox2* expression in the developing dorsal telencephalon.

Telencephalic specificity of transgene expression requires an 800nt fragment encompassing a nuclease hypersensitive site

Active, or potentially active transcriptional regulatory sequences often show hypersensitivity to DNAse I in native nuclei of cells that express the gene they control (Boyes and Felsenfeld, 1996).

We assayed for the presence of such DNAse I hypersensitive sites (HS) in the flanking sequences of the endogenous *Sox2* gene, using the strategy depicted in Fig. 3A. When nuclei of ES cells, treated with increasing amounts of DNAse I and subsequently digested (after deproteinization) with *Xho*I, were subjected to Southern analysis with probe 1, three DNAse I hypersensitive sites could be detected (Fig. 3B). The first one, HS1, was located within the DNA region critical for telencephalic expression on the basis of our functional analysis. Two additional sites (HS2 and HS3) were detected 3′ to the *Hin*dIII site, within a region that is still able to direct expression in ES cells, but not in the CNS.

To further refine the localization of HS1, DNAse I-treated ES cell nuclei were digested with *Hin*dIII, to generate a smaller restriction fragment encompassing HS1, and assayed with probes 2 and 3 (Fig. 3C and D). This allowed HS1 to be positioned as indicated in Fig. 3A. In control cells that do not express *Sox2*, such as A20 lymphoid cells, no DNAse I hypersensitivity was detected (Fig. 3E).

A related method to assay for accessibility of DNA sequences in intact nuclei relies on the use of restriction enzymes, rather than DNAse I, for chromatin digestion (Bresnik and Felsenfeld, 1994). ES cell nuclei were treated with *Pst*I, then deproteinized and further digested with *Hin*dIII, and finally analyzed by Southern blotting with probe 3 (Fig. 3F). A clear *Pst*I-*Hin*dIII band was observed, indicating that the *Pst*I site (indicated in Fig. 3A) was accessible in nuclei. This experiment independently defines a precise reference point for hypersensitivity, which is consistent with the DNAse I data. When the same experiment was carried out on nuclei of neural precursor cells (which express the endogenous *Sox2* gene, not shown) cultured from embryonic telencephalon (see later paragraphs), the *Pst*I site was equally or even more accessible than in ES cells (Fig. 4F). In contrast, in non-expressing cells (A20, Fig. 3F; STO fibroblasts, not shown) the *Pst*I site is not accessible.

HS 1 is the only nuclease hypersensitive site we detected in

the −5.7/−3.3 kb critical region; we thus asked whether a short DNA fragment encompassing HS 1 would be sufficient to confer telencephalic specific expression to the 3.3 *Sox2* 'minimal' promoter, which is inactive, by itself, in the CNS (Fig. 1C; Table 1). An 800 nt fragment encompassing HS 1 was linked to the 3.3 kb *Sox2* promoter upstream to the *β-geo* reporter, and assayed in transient transgenic embryos at midgestation (12.5-14 d.p.c.). Telencephalic-specific expression was observed, in the majority (5/9) of integrations analyzed, with a pattern superimposable to that observed with the 5.7 kb *Sox2* transgene; in three embryos, ectopic expression in surface ectoderm and/or hindlimbs was observed, together with weak telencephalic activity (Table 1).

Expression of the 5.7 kb *Sox2* transgene in CNS development and differentiation

To better characterize the gene expression pattern specified by the 5.7 kb *Sox2* 5′ flanking region during embryogenesis, stable transgenic lines were generated to assay for β-galactosidase expression at different developmental stages. Out of six founder mice tested, three generated progeny expressing the transgene in the telencephalon at 12.5 d.p.c.; one founder gave progeny showing ectopic transgene expression in the limbs (at 12.5 d.p.c.), and two founders gave non-expressing progeny (at 12.5 d.p.c.). Two of the lines expressing in the telencephalon were further characterized.

The expression pattern observed was qualitatively the same in independent transgenic lines. The expression levels could vary within the same line and within the same litter, both in absolute level and in the proportion of expressing cells; this effect was more pronounced in one of the two lines. These data indicate that the activity of this construct may be subject to position effect variegation of expression (Milot et al., 1996; Kioussis and Festenstein, 1997) and to variability of genetic background between different individuals.

β-galactosidase expression was already detectable, although at low level, in the inner cell mass of the blastocyst (3.5 d.p.c.; Fig. 4A) and at egg cylinder stages, in the embryonic ectoderm (6 d.p.c.; Fig. 4B). At gastrulation, transgene expression becomes excluded from the developing mesoderm and from the endoderm, and is progressively confined to the anterior part of the definitive ectoderm, the prospective neural plate, and to the neural plate itself (Fig. 4C,D, 7-7.5 d.p.c.; section in Fig. 5A). As anteroposterior patterning of the neuroepithelium becomes morphologically apparent, expression of the transgene is progressively lost posteriorly (Fig. 4E, 8.5 d.p.c.; 4F, 9.5 d.p.c.) until, at 10.5 d.p.c. (Fig. 4G) and subsequently (Fig. 4H, 12.5 d.p.c.; 4I, 14 d.p.c.), it is clearly confined to the developing telencephalic vesicles.

Within the telencephalon, at 12.5 d.p.c. (Fig. 5B), expression of the 5.7*Sox2β-geo* transgene is almost completely confined to the dorsal side, from which the cortex will develop. Some expression is also detected in the lateral ganglionic eminence (a site of strong expression of the endogenous *Sox2* gene, see Fig. 2D), indicating that the activity of the 5.7*Sox2β-geo* transgene is not strictly cortical specific. At 14.5 d.p.c. (Fig. 5C,E,F), β-gal staining is very intense in the ventricular zone, formed by the proliferating, still undifferentiated cells from

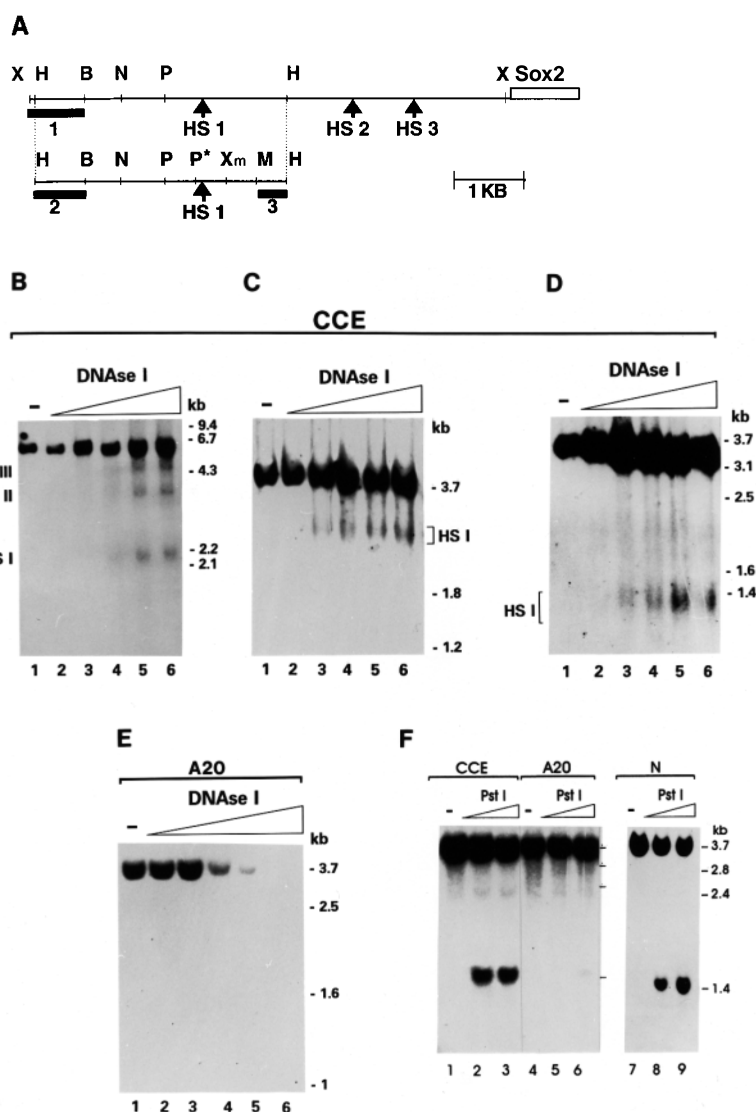


Fig. 3. Nuclease hypersensitivity in the Sox2 5′ flanking regions. (A) Diagram of the DNA region investigated. Boxes 1, 2 and 3 indicate probes. HS, DNAse I hypersensitive sites. The *Pst*I site preferentially cut in 3F is indicated by an asterisk. (B-E) DNAse I hypersensitivity. CCE ES cell (B,C,D), A20 lymphoid cell (E) nuclei were incubated in the absence (lane 1) or the presence of increasing amounts of DNAse I. The DNA was subsequently digested with X*ho*I (B) or *Hin*dIII (C-E) and subjected to Southern blot hybridisation with probe 1 (B), probe 2 (C) or probe 3 (D,E). Restriction enzyme digests of the cloned *Xho*I (B) or *Hin*dIII fragments (C-E) were run in parallel to locate the bands precisely (molecular mass indicated beside the panels). (F) *Pst*I sensitivity. CCE, A20 or neural precursor (neurosphere, N) cell nuclei were incubated in the absence, or the presence of 50 or 100 units of *Pst*I; DNA from these nuclei was further digested with *Hin*dIII, and hybridized to probe 3. H, *Hin*dIII; B, *Bgl*II; M, *Mlu*NI; N, *Nco*I; P and P*, *Pst*I; X, *Xho*I; Xm, *Xmn*I.

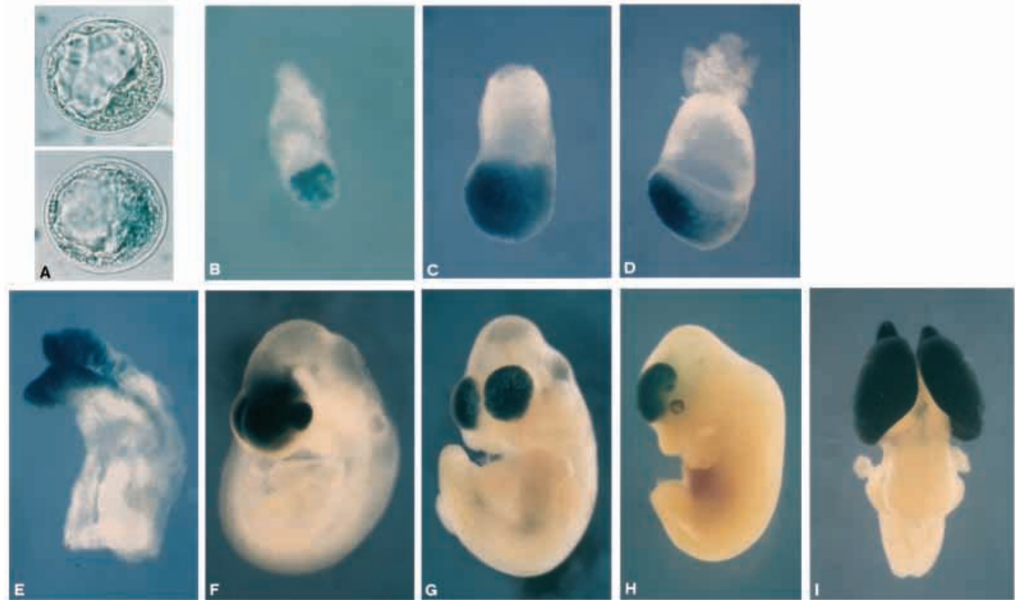


Fig. 4. β-gal activity in embryos from 5.7 kb *Sox2* transgenic lines. (A) 3.5 d.p.c. (lower panel, transgenic; upper panel, wild-type control). (B) 6 d.p.c. (C) 7 d.p.c. (D) 7.5 d.p.c. (E) 8.5 d.p.c. (F) 9.5 d.p.c. (G) 10.5 d.p.c. (H) 12.5 d.p.c. (I) 14 d.p.c., dissected brain.

which neurons and glial cells will arise. Expression rapidly fades in the outer intermediate and marginal zones, and is notably absent, or very decreased, in the first differentiating neurons in the cortical region (Fig. 5F, arrowheads), as well as in the hippocampal primordium in the medial wall of the ventricle (Fig. 5E).

At 17.5 d.p.c. (Fig. 5D), as cortical development proceeds, transgene expression is maintained in the progressively thinner ventricular/proliferative zone, predominantly in the medial, less developed region. In contrast, expression in the outer, differentiating layers is no longer detectable.

Interestingly, expression persists beyond embryonic development in the adult brain, where it is detected in the periventricular cells of the lateral ventricle (Fig. 5G-I) (notably including the ventral part), and along the rostral migratory stream reaching the olfactory bulb (not shown).

The 5.7 kb *Sox2* transgene is expressed in neural stem cells of the telencephalon

The ventricular zone of the telencephalon, where the 5.7 kb *Sox2β-geo* construct is highly expressed, is known to contain neural stem cells, in addition to proliferating precursors with more limited developmental options. Neural stem cells are endowed with high self-renewing potential, and are capable of giving rise to a progeny composed of the three major cell types in the CNS: neurons, astroglia and oligodendroglia (Reynolds and Weiss, 1992; Davis and Temple, 1994; McKay, 1997; Morrison et al., 1997; Stemple and Mahanthappa, 1997; Johansson et al., 1999; Doetsch et al., 1999). Functional in vitro assays of self-renewal and multipotentiality (see also Discussion) are essential to identify CNS neural stem cells as such, and to distinguish them from precursors with more limited developmental options (Morrison et al., 1999).

Stringent culture conditions that are permissive for neural stem cell proliferation and enrichment (stem cell medium; see Materials and Methods) have been developed previously; under these conditions, undifferentiated multipotent cells have been shown to proliferate, generating clones (neurospheres) that can be dissociated and replated in the same culture conditions, exponentially expanding, through serial passages, the native stem cell population and retaining the ability to differentiate into neuronal, oligodendroglial and astroglial derivatives, following mitogen removal (Reynolds and Weiss, 1992, 1996; Vescovi et al., 1993; Gritti et al., 1996; see also diagram in Fig. 6).

In order to assay whether the ventricular zone cells expressing the *Sox2* transgene include cells with functional stem cell properties, we established stem cell-enriched cultures from the telencephalon of 14.5 d.p.c. transgenic embryos (Reynolds and Weiss, 1992, 1996; Vescovi et al., 1993).

When cells isolated from the periventricular zone of the telencephalon of transgenic mice were grown in stem cell medium, neurospheres were generated (passage one neurospheres) and, after their dissociation into single cells, 20-90% of the cells stained for β-gal activity. This variability mirrors the variable levels of transgene expression observed by histological analysis in embryos. After several passages in culture, most of the uni- and bi-potential progenitor cells, with limited self-renewing/proliferative potential, have been lost from the population and the culture is enriched in the staminal component of the progenitor pool (Reynolds and Weiss, 1996; Gritti et al., 1996).

Exploiting the G418 resistance encoded by the *β-geo* transgene, we then selected for cells expressing the transgene by adding G418 to the culture (passage three neurospheres after dissociation to single cells, always in stem cell medium). At the minimum G418 concentration (200 μg/ml) which was lethal for non-transgenic control cells, cultures established from transgenic animals continued to proliferate exponentially through serial passaging, for at least three months, corresponding to more than 50 cell divisions. Under these conditions, more than 90% of individual neurospheres showed a large majority of blue cells (Fig. 6B). The resistance to G418 treatment indicates that cells that sustain the long-term expansion of the culture (i.e. the stem cells) continuously express the *Sox2*-G418 resistance gene (Fig. 6A).

To conclusively identify these transgene-expressing precursors as stem cells, we further evaluated essential features

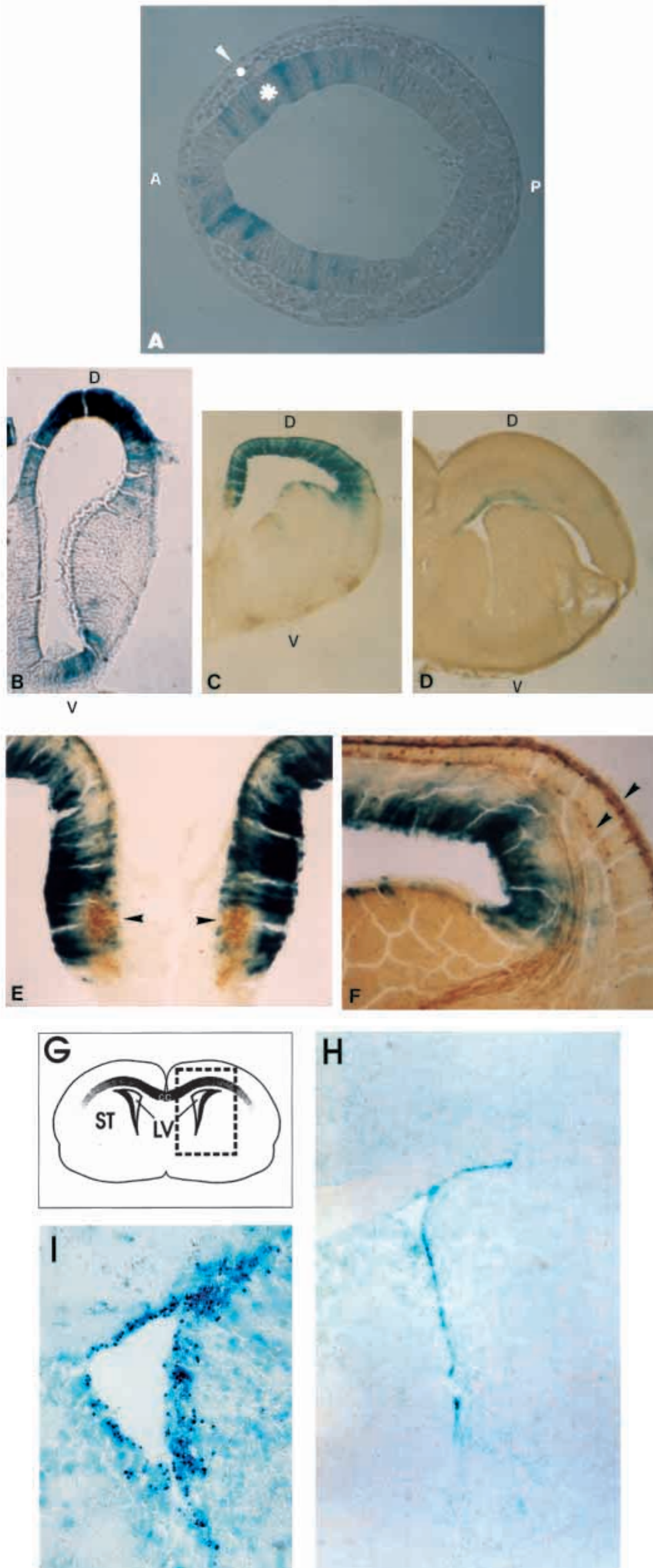


Fig. 5. β-gal activity of the 5.7 kb *Sox2* transgene in the developing neuroepithelium. (A) Transverse section through a 7.5 d.p.c. embryo. A, anterior; P, posterior; *, ectoderm; •, mesoderm; arrowhead, endoderm. (B-D) Coronal sections through the telencephalon at 12.5 d.p.c. (B), 14.5 d.p.c. (C) and 17.5 d.p.c. (D). D, dorsal; V: ventral. (E,F) Details of coronal sections of 14.5 d.p.c. telencephalon, stained for β-gal activity (blue) and with an anti-calretinin antibody (brown) recognizing differentiating neurons (arrowheads). (G-I) Coronal sections through adult telencephalon. H shows an area corresponding to the rectangle in the diagram in G. (I) Detail of the area surrounding the lumen of the ventricle (from an adjacent section, slightly more developed than H). LV, lateral ventricle; CC, corpus callosum; ST, striatum.

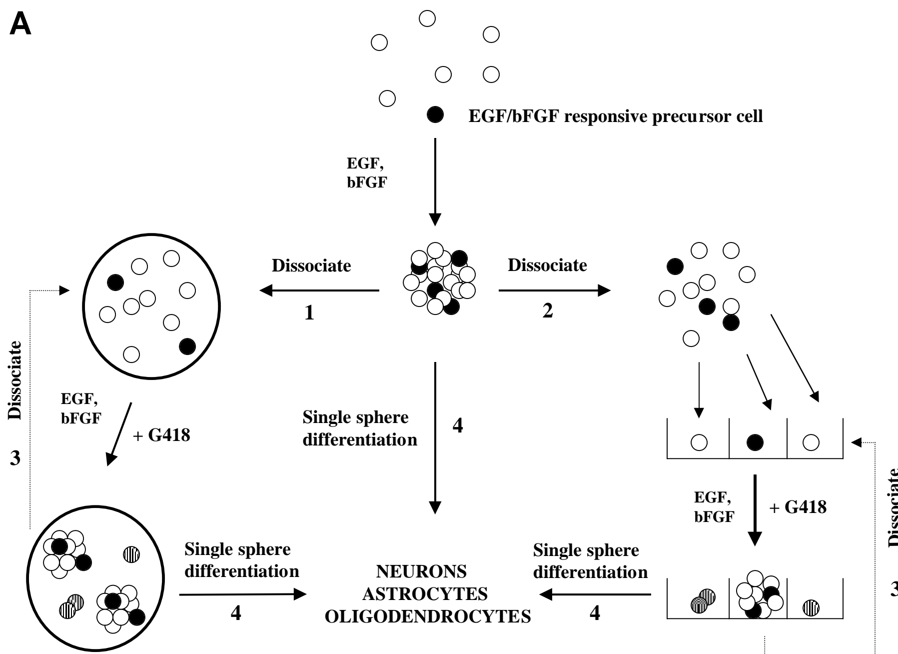
of staminality such as self-renewal and multipotentiality by means of a clonogenic assay (Gritti et al., 1996; Reynolds and Weiss, 1996; see Fig. 6A). Single cells dissociated from neurospheres at the third passage in G418 were plated at clonal density (see Materials and Methods) in stem cell medium (containing G418), following embedding in methylcellulose, to prevent cell migration and subsequent fusion of individual clones. Clones were formed; these were individually dissociated into single cells, which were replated into 24-well microplates under the same conditions (in G418). Numerous secondary clones were thus generated from the progeny of a single stem cell. These secondary spheres were either subcloned in the same conditions (stem cell medium with G418), giving rise to tertiary spheres, or differentiated in order to evaluate the simultaneous presence, within a single clonal sphere, of neuronal and astroglial/oligodendroglial antigens. Tertiary spheres were similarly differentiated. Neurons, astrocytes and oligodendrocytes, immunoreactive for MAP-2 and Tau-1, GFAP and O4 respectively, were detected within the differentiated progeny of each single (secondary and tertiary) clone (n=12; Fig. 6C-E), with percentages in accordance with previous findings (Reynolds and Weiss, 1992, 1996).

We conclude that the ventricular zone cell population that expresses the *Sox2* transgene includes cells with functional properties of neural stem cells, i.e. self-renewal and multipotentiality.

Telencephalic specificity of 5.7 kb *Sox2* transgene expression in stem cells

We wanted to investigate whether the observed expression of the *Sox2* transgene in telencephalic stem cells in culture really mirrors the telencephalic specificity of transgene expression observed in vivo, or whether it is rather the result of non-specifically 'permissive' culture conditions, such as growth factor stimulation or other. Further, we wanted to evaluate whether telencephalic transgene-expressing cells represented a significant proportion of the total telencephalic stem cells.

To investigate these points, stem cell-enriched (neurosphere) cultures were established from the dorsal telencephalon and from the spinal cord (as control) of transgenic embryos, and maintained in parallel for extended periods of time (up to two/three months) in the absence of G418, thus avoiding any selective pressure. To quantitatively estimate the proportion of G418-resistant (and thus transgene-expressing) stem cells in the two cultures, aliquots of cells were removed from the cultures at successive time points, and dissociated; half was plated (at clonal density) in the absence, and half in the presence of G418, and grown to form neurospheres. Reynolds and Weiss (1996) and Gritti et al. (1996) previously showed that only cells with extended self-renewing potential are able to form neurospheres under these conditions; moreover, multipotentiality is retained in



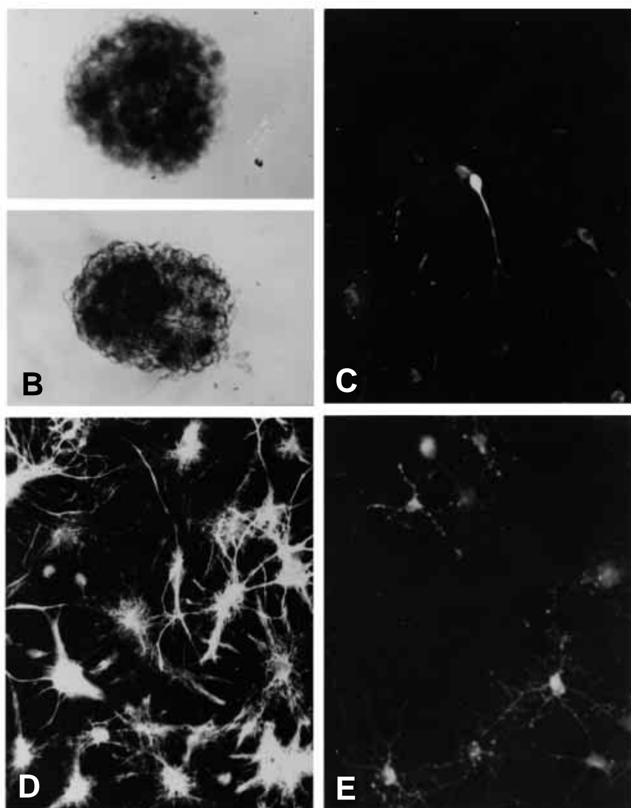


Fig. 6. (A) Primary cells expressing the β-geo/G418 resistance *Sox2* transgene include precursor cells which are both self-renewing and multipotent. When dissociated transgenic primary cells from 14.5 d.p.c. dorsal telencephalon are exposed to EGF and bFGF, large spheres of undifferentiated cells are formed (primary neurospheres). Upon dissociation of primary neurospheres into single cells and replating (in populations, 1, or at clonal density, 2), in the presence of G418, a proportion of the cells (black circles) will form secondary spheres. These can be further dissociated to single cells and replated (3), and will give rise to tertiary spheres, and so on. Dissociation of individual neurospheres always gives rise to multiple neurospheres upon replating (10-20% of cells in a sphere will generate new clones), resulting in a progressive expansion of the clone-forming founder cells. Other cells (white circles), with more limited proliferative potential, eventually differentiate or die (hatched circles) without forming neurospheres. Differentiation of primary, as well as of secondary and tertiary neurospheres (4) results in the production of neurons, astrocytes and oligodendrocytes (in 90-100% of the spheres), demonstrating multipotentiality of clone-forming cells, and its maintenance through passaging (adapted from Reynolds and Weiss, 1996). Expansion of multipotential neurosphere-forming cells in the presence of G418 demonstrates that these cells continuously express the transgene. (B-E) Clonal neurospheres derived from G418-selected transgenic brain cells, and differentiation of cells belonging to a single representative neurosphere. (B) Two clonal neurospheres grown in stem cell medium with G418, stained for β-gal activity. (C-E) Cells differentiated from a clonal neurosphere and stained for the presence of neuronal (C, MAP-2 and Tau-1), astroglial (D, GFAP) or oligodendroglial (E, O4) antigens.

this system in the large majority of clone-forming cells even at late passages. On this basis, we consider that the proportion of neurospheres formed in the presence of G418 versus neurospheres formed in the absence of G418 represents the proportion of transgene-expressing stem cells.

A representative experiment is shown in Fig. 7. A high proportion of telencephalic stem cells were G418 resistant, already at early passages (Fig. 7A; see Materials and Methods); this proportion showed little decline over two months in culture (Fig. 7A; over 35 cell divisions, and 9 dissociation passages). In contrast, no G418-resistant clones were obtained from the spinal cord cultures (n=0/400, see Materials and Methods) at any time point. Essentially identical results were obtained in cultures from low level expressing litters, where the proportion of G418-resistant telencephalic stem cells was about 20-30% at the beginning of the culture (not shown).

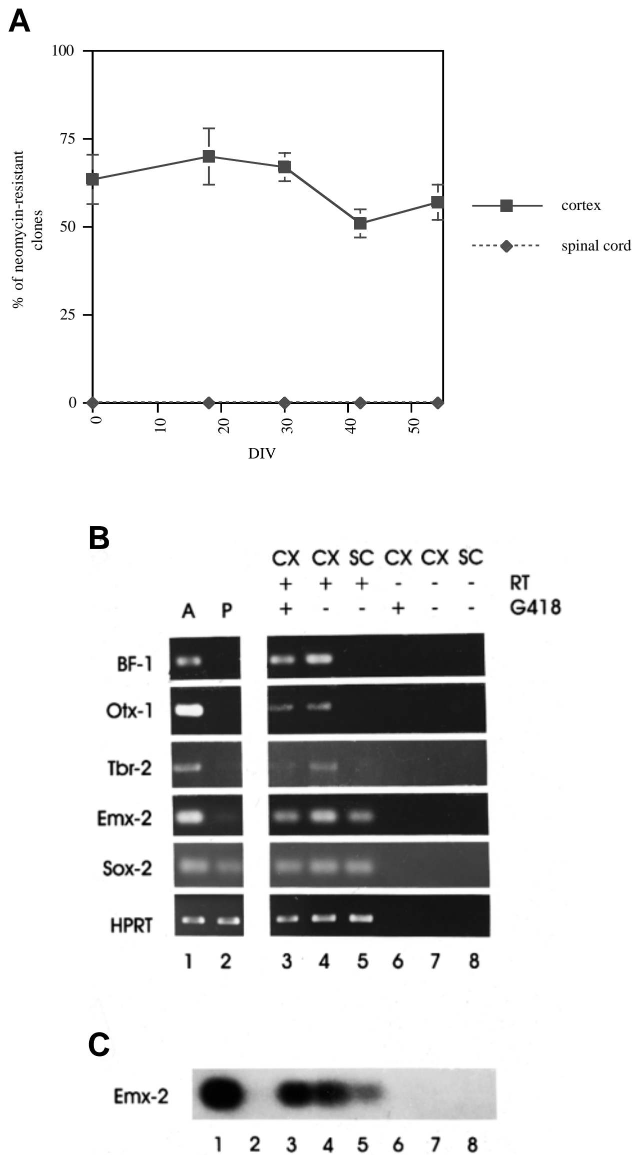


Fig. 7. (A) G418 resistance of transgenic dorsal telencephalic, or spinal cord, neural stem cells during long-term growth in the absence of selective pressure (G418). Cultures were grown in the absence of G418 for extended periods of time, and aliquots were assayed, at successive time points, for neurosphere (clone) forming ability in a clonogenic assay. Each point in the graph represents the mean percentage of neurospheres formed in the presence of G418, relative to those formed in the absence of G418, averaged on the count of six or more independent wells. Between 300 and 600 clones were obtained, in the absence of G418, from both telencephalic and spinal cord cultures, corresponding to a plating efficiency of 10-20%. Standard errors are indicated by vertical bars. DIV, days in vitro; circles, cortex cultures; triangles, spinal cord cultures. (B,C) RT-PCR analysis of expression of telencephalic-restricted transcription factor genes in transgenic neurospheres from dorsal telencephalon (unselected, or G418 selected), or spinal cord. CX, cortex; SC, spinal cord; RT, reverse transcriptase. (B) cDNAs generated by reverse transcription of RNA from passage 12 neurospheres were amplified with primers specific for the cDNAs of the genes indicated on the left, for 30 or 35 (Tbr-2) cycles (lanes 3-8). Lanes 1 and 2 show assays (same cycle numbers) on RNA extracted from telencephalon (A) or spinal cord region (P) of 14.5 d.p.c. embryos, for reference. Lanes 3, 4 and 5 show RT-PCR assays on cortex- or spinal cord-derived neurospheres (as indicated above the lanes); lanes 6, 7 and 8 are the PCRs carried out using the reverse transcriptase-negative control reactions. Endogenous Sox2 expression (which is panneural in the undifferentiated neuroepithelium; see Results and Discussion) is comparatively assayed. The housekeeping gene HPRT is used as standard for the use of similar amounts of cDNA in each reaction (Keller et al., 1993; see Materials and Methods). (C) PCR for *Emx2* performed as in A, but only for 22 cycles, to compare expression levels in the exponential phase of the PCR reaction; samples were transferred to a filter and hybridized with an *Emx2* specific probe. Samples in lanes 1-8 are in the same order as in B.

We conclude that expression of the transgene in telencephalic stem cells in vitro reflects the regional specificity observed in vivo, and is not the artifactual result of the culture conditions. Moreover, the expression of the transgene is relatively stable (even in the absence of selective pressure) in long-term cultures.

Of note, most, but not all of the telencephalic stem cells express the transgene in the culture assay (Fig. 7A). Thus, we are not able to rule out that some telencephalic stem cells in fact escape 'regionalization' of transgene expression. However, it is also possible that the failure of some stem cells to express the transgene simply reflects position effect variegation of transgene expression within the cell population in vivo, a common finding in transgenic experiments (Milot et al., 1996; Kioussis and Festenstein, 1997).

The question arises whether regionally expressed endogenous genes show, in stem cell-enriched cultures in vitro, the same regional specificity that they exhibit in vivo, as we observe with the *Sox2* transgene. To verify this point, we assayed for the expression, in neurospheres, of various endogenous transcription factor-encoding genes, known to be expressed in the dorsal telencephalic ventricular zone at 14.5 d.p.c. (see Fig. 7B,C). Spinal cord- and cortex-derived neurospheres from the same experiment described above (Fig. 7A), grown both in the absence or the presence of G418 for 55 days, were analyzed by RT-PCR (30-35 cycles; Fig. 7B,C). Whereas panneural genes such as endogenous *Sox2* (Fig. 7 and data not shown) are expressed, as expected, in both spinal cord- and cortex-derived neurospheres, several dorsal telencephalic genes (*BF1,* Xuan et al., 1995; *Otx1,* Simeone et al., 1993, Frantz et al., 1994; *Tbr2*, Bulfone et al., 1999) are expressed specifically in cortex-, but not in spinal cord-derived neurospheres (with the exception of *Emx2*, Simeone et al., 1992). The absence of expression of *BF1*, *Otx1* and *Tbr2* in spinal cord neurospheres was confirmed at 40 cycles; the identity of the amplified bands was confirmed by hybridization to radioactive probes (not shown).

We conclude that both the *Sox2* transgene and various (even if not all) endogenous telencephalic genes (Fig. 7) retain regional specificity of expression throughout long-term in vitro neurosphere culture.

Emx2 is an apparent exception, as spinal cord neurospheres express *Emx2* at levels only moderately lower than those observed in cortical neurospheres (Fig. 7B,C). Although *Emx2* was reportedly not detectable in spinal cord by in situ hybridization (Simeone et al., 1992), we consistently detect low levels of *Emx2* by RT-PCR (Fig. 7) and long exposures of northern blot (not shown) in spinal cord RNA. It is possible

that *Emx*2-expressing cells are poorly represented in the spinal cord, but not in stem cell-enriched cultures; alternatively, *Emx2* transcription could be negatively regulated by environmental factors.

Interestingly, *Tbr*2 is expressed at higher levels in unselected, versus G418 selected neurospheres. This result presumably reflects the cellular heterogeneity of *Sox2* transgene expression within the neurospheres (see Fig. 6); we hypothesize that cells expressing low levels of, or no, β-geo (possibly the less immature component, see Fig. 5) might be enriched in transcripts of *Tbr2* relative to other, more immature, precursors. Indeed, *Tbr2* expression is initially localized mainly within the mantle zone, and progressively shifts to the ventricular zone only during late development (Bulfone et al., 1999).

DISCUSSION

We identified flanking sequences of *Sox2*, a panneurally expressed gene, which drive reporter gene expression to the dorsal part of the developing telencephalon, a subregion of the entire *Sox2* expression domain. These sequences are also necessary, within the endogenous gene, for expression in the dorsal telencephalon.

By exploiting the ability of our construct to give G418 resistance, we further showed by clonal analysis and long-term culture that the *Sox2* flanking sequences direct gene expession to neural stem cells (and precursors) of the telencephalon, but not to stem cells of the spinal cord. This specificity is largely maintained through extensive in vitro serial passaging. Thus, region-specific gene expression within the embryonic neural tube is mirrored by a similar specification at the level of functionally defined neural stem cells, which persists through long-term in vitro culture.

Sox2 regulatory regions critical for telencephalic gene expression

We directed the expression of a *β-geo* transgene to the developing telencephalon (Figs 1 and 4) and in particular to its dorsal part (Fig. 5B-D). 5′ regulatory sequences of *Sox2* down to −5.7 kb are able to drive dorsal telencephalic transgene expression; the telencephalic activity is critically dependent on DNA sequences located between −5.7 and −3.3 kb of the mouse *Sox2* promoter (Table 1; Fig. 1).

The region-specific expression of the transgene is somewhat unexpected. In fact, *Sox2* is expressed (in undifferentiated cells) along the entire developing neural tube (summarized in Uchikawa et al., 1999).This suggests that panneural expression of *Sox2* at advanced stages (after 8.5-10.5 d.p.c., see Fig. 4) results from the joint action of different regulatory elements, some of which (e.g. our telencephalic element) act predominantly in specific regions.

The existence, within a single gene, of different regulatory regions that specify gene expression in distinct sectors of the developing CNS, was previously demonstrated, in domains in the posterior CNS, by work on the *Hox* genes (Marshall et al., 1994; Studer et al., 1994). In addition, mutations in a nuclear hormone receptor site within the enhancers of two panneurally expressed genes, *nestin* and *B-FABP*, decrease expression of a linked reporter gene within the telencephalic (and mesencephalic) region, but not at more posterior locations (Josephson et al., 1998). Our experiments confirm the importance of specific regulatory elements for telencephalic expression in the context of a panneurally expressed gene. However, whereas with the *nestin* and *B-FABP* enhancers, activity of the transgene in the telencephalon could not be observed separately from general panneural activity, the *Sox2* regulatory element is active specifically in the telencephalon in the absence of activity elsewhere.

The existence of telencephalic-specific regulatory elements in panneural genes might reflect the need of the cells to respond to signals specific to this area. Specific signals are indeed involved in the induction and development of the most anterior neuroectoderm (Lumsden and Krumlauf, 1996; Shimamura and Rubenstein, 1997; Beddington and Robertson, 1998; Rubenstein et al., 1998; Ruiz i Altaba, 1998). Thus, *Sox2* might be under the control of different signals in the anterior, as opposed to the posterior regions.

The functional relevance within the endogenous gene of putative regulatory elements has rarely been confirmed by homologous recombination-mediated deletion (Studer et al., 1998; Gavalas et al., 1998; Herault et al., 1998). Notably, regulatory regions which were thought to be essential for expression on the basis of transgenic analysis, were shown to be partially, or even fully dispensable when assayed in the context of the endogenous gene (Fiering et al., 1995; McDevitt et al., 1997; Epner et al., 1998; Higgs, 1998). In chimaeras at 12.5 d.p.c., the deletion of the *Sox2* −5.7 to −3.3 kb region greatly decreased (Fig. 2) expression in the dorsal telencephalon relative to the ventral telencephalon. This confirms the critical importance for dorsal telencephalic expression of the −5.7 to −3.3 kb region of the endogenous *Sox2* gene. A weaker effect of the deletion was also observed in more posterior regions such as mesencephalon and diencephalon; this was not predicted by the transgenic experiments. This suggests that, within the endogenous *Sox2* gene, the −5.7/−3.3 kb region might synergize, in the mesencephalon and diencephalon, with other elements that are not represented within the transgene. Additionally, the observation that ES cells in which the −5.7/−3.3 kb region was deleted express the *β-geo* gene inserted in the endogenous *Sox2* locus equally as well as non-deleted cells, further suggests that in ES cells the −5.7 to −3.3 kb region is functionally redundant.

The *Sox2* transgene is expressed in neural stem cells of the telencephalon

Sox2 expression, which is uniformly present in the undifferentiated neuroepithelium from neural plate stages, is down-regulated in cells undergoing differentiation, and becomes restricted to the ventricular zone following the onset of neural tube differentiation (Collignon, 1992; Uwanogho et al., 1995).

The similar localization of the 5.7 kb *Sox2β-geo* transgene activity to ventricular zone cells of the embryo (Fig. 5C,D) and to periventricular cells of the adult brain (Fig. 5G-I), suggests that the transgene is expressed in undifferentiated neural precursor cells; among these precursors are neural stem cells (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992; Davis and Temple, 1994; Gritti et al., 1996; McKay, 1997; Johansson, 1999; Doetsch et al., 1999; Temple and Alvarez-Buylla, 1999).

Is the *Sox2* transgene already expressed in the stem cell, or in committed precursors only?

According to a widely accepted definition (Loeffler and Potten, 1997), stem cells are identified, by functional attributes, as (i) undifferentiated cells, (ii) cells capable of proliferation, (iii) self-renewing cells (i.e. with the ability to generate, through symmetrical divisions, daughter cells displaying the same functional properties of the parent cell) and (iv) cells capable of generating a wide array of differentiated progeny.

However, as has been pointed out (Gage, 1998), no real number of cell divisions has been set as a criterion for defining the self-renewing ability of a true stem cell. So we are left with the "overly inclusive definition that any cell that can divide more than once, or some indetermined number of times, and that can give rise to cells that exhibit the most primitive of phenotypes for neurons, astrocytes and oligodendrocytes can be called a CNS stem cell" (Gage, 1998).

Indeed, Johe et al. (1996) showed that up to 40% of freshly dissociated 14 d.p.c. cortical CNS cells are able to replicate for at least a few times and to give rise to neurons, astrocytes and oligodendrocytes; on these grounds, they proposed stem cells to be fairly abundant in the fetal brain. On the other hand, when cells are grown (at clonal densities) for extended periods of time, only 0.3-3% of the cells display long-term self-renewal and multipotentiality (Davis and Temple, 1994; Reynolds and Weiss, 1996; Gritti et al., 1996; Tropepe et al., 1999). Based on the latter observations, Davis and Temple (1994) proposed that "a minor stem cell population coexists with a larger population of apparently more restricted progenitors, and that multipotential stem cells form the foundation of the cerebral cortex: self-renewing to produce and maintain cortical germinal layers, and dividing asymmetrically to generate different restricted progenitors that amplify individual cortical cell types".

An important role for stem cell proliferation in CNS development is suggested by the severe effects on nervous system development (and stem cell proliferation; Tropepe et al., 1999), caused by the knock-out of genes encoding the receptors for bFGF or EGF (Deng et al., 1994; Yamaguchi et al., 1994; Threadgill et al., 1995; Sibilia et al., 1998) which transduce mitogenic signals in CNS stem cells.

In our experiments, we defined neural stem cells on the basis of the restrictive criterion of *extended* self-renewal in culture and multipotentiality. Here we have shown that cells continuously expressing the *Sox2β-geo* transgene and possessing the above stem cell characteristics can be isolated from the embryonic telencephalon. In fact, a phenomenon that is strictly stem cell-driven, such as the exponential growth of the cell number in culture conditions specifically designed for stem cell growth and enrichment, is unaffected by the presence of lethal doses of G418 in cultures from transgenic animals carrying the neomycin/G418-resistance gene under the control of the *Sox2* promoter (Fig. 6). The conclusive demonstration of the staminal nature of the *Sox2β-geo* transgene-expressing cells in embryonic telencephalic cultures is provided by a standardized serial subcloning procedure previously used to identify neural crest and adult CNS stem cells (Stemple and Anderson, 1992; Reynolds and Weiss, 1996; Gritti et al., 1996) (see diagram in Fig. 6). We showed that single G418-selected precursors (i) could generate (always in G418) clones that contained secondary precursor cells which could, in turn, generate new clones – and therefore possessed self-renewal; (ii) had the ability to give rise to differentiated progeny comprising neurons, astrocytes and oligodendrocytes (Fig. 6) – and thus qualified as multipotential elements.

Transgene-expressing cells of this kind could also be isolated from adult brain, indicating that the activity of the *Sox2* regulatory sequences in telencephalic stem cells is not limited to embryonic development (our unpublished data).

We conclude that neural stem cells from embryonic telencephalon express the *Sox2* transgene throughout their in vitro long-term growth and we propose that this reflects a property already present in vivo (see below).

This conclusion does not imply that *Sox2* transgene expression is stem cell specific. In fact, neurospheres consist of up to 10-20% of neural stem cells (Reynolds and Weiss, 1996; Gritti et al., 1996), and a majority of proliferating precursors with more limited self-renewal ability and more restricted fates. Nevertheless, over 90% of the cells in the neurospheres (in G418) stain for β-gal activity, indicating that the transgene is also expressed in early precursors devoid of stem cell properties.

Regional specificity of *Sox2* transgene-expressing stem cells and maintenance of stem cell gene expression programs

An important question is at what cellular stage does CNS patterning arise in development (Panchision et al., 1998; Edlund and Jessell, 1999). Is patterning already established in stem cells, generating regional heterogeneity among these cells, or in precursors downstream to the stem cell? Further, once established, how is patterning maintained during the multiple cell divisions occurring in embryogenesis and to what extent is it reversible in response to progressive modifications of the environment?

We started to address some of these questions directly at the level of functionally defined stem cells grown in vitro, by investigating their *Sox2* transgene-expression program (together with that of region-specific endogenous genes) and in particular the possibility that these programs differ between stem cells from different CNS regions.

Previous work along these lines was carried out on primary unfractionated neuroepithelial cells grown in vitro on a short-term basis (Robel et al., 1995; Nakagawa et al., 1996), and indicated that these cells may maintain, for a few days, differential expression patterns of region-specific genes. However, to our knowledge, no studies have appeared of non-immortalized, long-term proliferating, neural stem cells.

Our experiments show that cells with functional properties of neural stem cells, cultured from different CNS regions (dorsal telencephalon/spinal cord) at 14.5 d.p.c., are regionally specified with respect to expression of the *Sox2* transgene (and thus to the network of transcription factors driving its expression; Figs 6, 7); further, this regional specificity is largely maintained (even in the absence of G418 selective pressure) through extended in vitro stem cell culture (Fig. 7), which involves multiple stem cell divisions and repeated dissociations to single cells (Reynolds and Weiss, 1996; Gritti et al., 1996).

In this system, after the original acute tissue dissociation at plating, rapid expansion of stem cells occurs in response to mitogen stimulation, and is coupled with the differentiation

and loss of committed progenitors originally present (Reynolds and Weiss, 1996; Gritti et al., 1996). These processes, through serial passaging of the culture, are expected to lead to early dilution and loss of any diffusible factors and cell-cell interactions originally present in the embryo, that might have affected stem cell gene expression. The persistence of a large proportion of stem cells expressing the G418 resistance gene after about two months in culture (Fig. 7A) strongly suggests that their transgene expression program is stably inherited in the absence of external cues provided by the embryonic environment.

The slight decline of expressing cells observed during the late stages of the culture (Fig. 7A) is unlikely to reflect the loss of environmental factors, as this loss is predicted to occur already at the very early stages of the culture. We favour an alternative explanation: randomly integrated genes are known to become repressed by the encroachment of surrounding chromatin silencing effects on their regulatory regions (Walters et al., 1996; Pikaart et al., 1998).

The results obtained with the *Sox2* transgene (Fig. 7A) are consistent with those obtained (on the same neurospheres) by RT-PCR analysis of the expression of various transcription factor-encoding genes (Fig. 7B,C).

Indeed, the expression of the telencephalic genes encoding BF1, Otx1, Tbr2 (Fig. 7) and Otx2 (not shown) is observed in telencephalic, but not in spinal cord neurospheres, after 12 passages (over 2 months) in culture. This experiment does not prove that these particular genes are expressed in the stem cell component of the neurosphere, as opposed to the more mature precursors. Nevertheless, as neurospheres are generated from stem cells, long-term differences in gene expression between neurospheres cultured from different regions must derive from persisting regional heterogeneity among stem cells themselves.

In conclusion, our in vitro findings imply that, in vivo, at least a proportion of cortical stem cells, at 14.5 d.p.c., might be endowed with region-specific gene expression programs (best exemplified by the *Sox2* transgene) that, once acquired, are transmissible to their progeny through divisions expanding the stem cell population, in the absence of cues provided by the embryonic environment.

This suggests that, during embryonic development, stem cells might acquire, through the initial action of extracellular signals, region-specific patterns of gene expression, which can then be inherited for at least a number of stem cell divisions, even when the original signal is no longer in existence.

Such a strategy may be particularly relevant in the cerebral cortex/telencephalon, where regional differentiation and neurogenesis occur at a significantly late developmental stage, and thus a much larger number of cell divisions are required, prior to differentiation, than in the more caudal neural tube (Tanabe and Jessell, 1996).

Neural cells, when transplanted into the CNS in heterotopic areas, can acquire phenotypic characteristics typical of the new location (reviewed by Barbe, 1996; Panchision et al., 1998; McKay, 1999). In addition, clones of neural stem cells (obtained exactly as in this paper) are exceptionally plastic, as they can even repopulate the hematopoietic system of irradiated mice (Bjornson et al., 1999).

Is the acquisition of inheritable gene expression programs (as shown in Fig. 7, and discussed above) necessarily in conflict with stem cell plasticity? We believe not. It is possible that stem cells, during their normal development, acquire inheritable patterns of gene expression, while maintaining the potential to respond to new local signals, and to further specify their gene expression programs during the progressive sub-regionalization of the CNS, according to their location in the developing nervous system. Thus, upon transplantation to new environments, stem cells (and/or their immediate descendants) might acquire new characteristics.

In our experiments, we removed cells from their original telencephalic environment, demonstrating maintenance of regional programs through passaging in vitro, but we did not challenge them with signals from other regions, to test for plasticity.

In addition, plasticity of at least one gene expression program is suggested by our own experiments; in fact, *Emx2*, which is expressed at extremely low levels in the spinal cord in vivo, is 'induced' in stem cell cultures to levels comparable to those observed in cortex-derived cultures, suggesting that the *Emx2* expression program can indeed be subject to negative environmental control in the spinal cord.

The availability of G418-selected telencephalic stem cells, as well as of transgenic spinal cord cells, may enable us to probe the plasticity of transgene expression when reintroduced into different regions of the nervous system (e.g. Barbe, 1996; Cohen-Tannoudji et al., 1994; Gitton et al., 1999; McKay, 1999).

Neural stem cell transplantation gives great hope for the treatment of human brain injury and disease. The regional specificity of stem cells shown in this paper may have to be borne in mind when planning treatments of this kind. Further, in vitro drug selection of cells expressing resistance genes under the control of regulatory regions such as our 5.7 kb promoter may provide a way to generate lines enriched in stem cells, versus differentiating progenitors (see also McWhir et al., 1996). Neuronal precursors were indeed enriched by a strategy similar to that presented here in differentiating ES cell lines treated with retinoic acid (Li et al., 1998). Similar approaches might hopefully lead to the generation of human stem cell lines with region-specific properties.

This work was supported by Telethon grant D27 to S. N., and by a MURST 40% grant to R. Taramelli; R. G. and A. V. were supported by EEC grant Biomed 4-98-0159; R. L.-B., and initial parts of this work, were supported by the MRC. We wish to thank Laura Pozzi for much help and discussion, Sam Weiss for helpful comments, Alessandro Bulfone for sharing unpublished results prior to publication, and Ron McKay for insightful advice. We are also grateful to Daniela Santoni for expert animal care, Paola Ricciardi-Castagnoli and the Department of Pharmacology for generously allowing us the use of the microinjection set-up and for hospitality to our mice, Laura Tatangelo for help with transgenic work, and Mario Azzini for photographic assistance.

REFERENCES

Barbe, M. F. (1996). Tempting fate and commitment in the developing forebrain. *Neuron* **16**, 1-4.

Beddington, R. S. P. and Robertson, E. J. (1998). Anterior patterning in mouse. *Trends Genet*. **14**, 277-284.

Bjornson, C. R. R., Rietze, R. L., Reynolds,B. A., Magli, C. and Vescovi, A. L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**, 534-537.

Boyes, J. and Felsenfeld, G. (1996). Tissue-specific factors additively increase

the probability of the all-or-none formation of a hypersensitive site. *EMBO J.* **15**, 2496-2507.

Bradley, A. and Liu, P. (1996). Target practice in transgenics. *Nature Genet.* **14**, 121-123.

Bresnik, E. H. and Felsenfeld, G. (1994). Dual promoter activation by the human β-globin locus control region. *Proc. Natl. Acad. Sci. USA* **91**, 1314-1317.

Bulfone, A., Martinez, S., Marigo, V., Campanella, M., Basile, A., Quaderi, N., Gattuso, C., Rubenstein, J. L. R. and Ballabio, A. (1999). Expression pattern of the Tbr2 (Eomesodermin) gene during mouse and chick brain development. *Mech. Dev.* **84**, 133-138.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem*. **162**,156-159.

Cohen-Tannoudji, M., Babinet, C. and Wassef, M. (1994). Early determination of a mouse somatosensory cortex marker. *Nature* **368**, 460-463.

Collignon, J. (1992). *Study of a New Family of Genes Related to the Mammalian Testis Determining Gene*. PhD thesis, CNAA London.

Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P. N. and Lovell-Badge, R. (1996). A comparison of the properties of *Sox-3* with *Sry* and two related genes, *Sox-1* and *Sox-2*. *Development* **122**, 509-520.

Davis, A. A. and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* **372**, 263-266.

Deng, C-X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M. and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev*. **8**, 3045-3057.

Doetsch, F., Caillé, I., Lim, D. A., Garcìa-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.

Edlund, T. and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: A view from the nervous system. *Cell* **96**, 211-224.

Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M. A., Fiering, S., Enver, T., Martin, D. I. K., Kennedy, M., Keller, G. and Groudine, M. (1998). The β-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse β-globin locus. *Mol. Cell*. **2**, 447-455.

Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D.I.K., Enver, T., Ley, T.J. and Groundine, M. (1995). Targeted deletion of 5'HS2 of the murine b-globin LCR reveals that it is not essential for proper regulation of the β-globin locus. *Genes Dev*. **9**, 2203-2213.

Fonseca, M., Del Rio, J. A., Martinez, A., Gomez, S. and Soriano, M. (1995). Development of calretinin immunoreactivity in the neocortex of the rat. *J. Comp. Neurol*. **361**, 177-192.

Forrester, W. C., Epner, E., Driscoll, C., Enver, T., Brice, M., Papayannopoulou, T. and Groudine, M. (1990). A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin locus. *Genes Dev.* **4**, 1637-1649.

Frantz, G. D., Weimann, J. M., Levin, M. E. and McConnell, S. K. (1994). Otx-1 and Otx-2 define layers and regions in developing cerebral cortex and cerebellum. *J. Neurosci.* **14**, 5725-5740.

Friedrich, G. and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513-1523.

Gage, F. (1998). Stem cells of the central nervous system. *Curr. Op. Neurobiol.* **8**, 671-676.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F. M., Krumlauf, R. and Chambon, P. (1998). Hoxa1 and Hoxb1 synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Gitton, Y., Cohen-Tannoudji, M. and Wassef, M. (1999). Specification of somatosensory area identity in cortical explants. *J. Neurosci*. **19**, 4889-4898.

Gritti, A., Parati, E. A., Cova, L., Frolichstahl, P., Galli, R., Wanke, E., Faravelli, L., Morasutti, D. J., Roisen, F., Nickel, D. D. and Vescovi,A. L. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci*. **16**, 1091-1100.

Herault,Y., Beckers, J., Kondo, T., Fraudeau, N. and Duboule, D. (1998). Genetic analysis of a Hox-12 regulatory element reveals global versus local modes of control of the HoxD complex. *Development* **125**, 1669-1677.

Higgs, D. (1998). Do LCRs open chromatin domains? *Cell* **95**, 299-302.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo. A Laboratory Manual*. 2nd. ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Jacobson, M. (1991). *Developmental Neurobiology* 3rd ed. New York, NY: Plenum Press.

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U. and Frisén, J. (1999). Identification of a neural stem cell in the mammalian central nervous system. *Cell* **96**, 25-34.

Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic,M. M. and McKay, R. D. G. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev*. **10**, 3129-3140.

Josephson, R., Muller, T., Pickel, J., Okabe, S., Reynolds, K., Turner, P. A., Zimmer, A. and McKay, R. D. G. (1998). POU transcription factors control expression of CNS stem cell-specific genes. *Development* **125**, 3087-3100.

Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M. V. (1993). Hematopietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol*. **13**, 473-486.

Kioussis, D. and Festenstein, R. (1997). Locus control regions:overcoming heterochromatin-induced gene inactivation in mammals. *Curr. Opin. Gen. Dev*. **7**, 614-619.

Li, M., Pevny, L., Lovell-Badge, R. and Smith, A. (1998). Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr. Biol.* **8**, 971-974.

Loeffler, M. and Potten, C. S. (1997). Stem cells and cellular pedigrees -a conceptual introduction. pp 1-27 In *Stem Cells*. (ed. C. S. Potten). San Diego (CA): Academic Press.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1. Nature* **370**, 567-571.

McDevitt, M. A., Shivdasani, R. A., Fujiwara, Y., Yang, H. and Orkin, S. H. (1997). A 'knockdown' mutation created by cis-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. *Proc. Natl. Acad. Sci. USA* **94**, 6781-6785.

McKay, R. (1997). Stem cells in the central nervous system. *Science* **276**, 66-71.

McKay, R. (1999). Brain stem cells change their identity. *Nature Med*. **5**, 261-262.

McWhir, J., Schnieke, A. E., Ansell, R., Wallace, H., Colman, A., Scott, A. R. and Kind, A. J. (1996). Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background. *Nature Genet.* **14**, 223-226.

Milot, E., Strouboulis, J., Trimborn, T., Wijgerde, M., de Boer, E., Langeveld, A., Tan-Un, K., Vergeer, W., Yannoutsos, N., Grosveld, F. and Fraser, P. (1996). Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* **87**, 105-114.

Mizuseki, K., Masashi, K., Matsui, M., Nakanishi, S. and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**, 579-587.

Morrison, S. J., Shah, N. M. and Anderson, D. J. (1997). Regulatory mechanisms in stem cell biology. *Cell* **88**, 287-298.

Morrison, S. J., White P. M., Zock C. and Anderson, D. J. (1999). Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**, 737-749.

Nakagawa, Y., Kaneko, T., Ogura, T., Suzuki, T., Torii, M., Kaibuchi, K., Arai, K., Nakamura, S. and Nakafuku, M. (1996). Roles of cell-autonomous mechanisms for differential expression of region-specific trascription factors in neuroepithelial cells. *Development* **122**, 2449-2464.

Panchision, D., Hazel, T. and McKay, R. (1998). Plasticity and stem cells in the vertebrate nervous system. *Curr. Opin. Cell Biol*. **10**, 727-733.

Pikaart, M.J., Recillas-Targa, F. and Felsenfeld, G. (1998). Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacitylation and is prevented by insulators. *Genes Dev.* **12**, 2852-2862

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710.

Reynolds, B. A., Tetzlaff, W. and Weiss, S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci*. **12**, 4565-4574.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Robel, L., Ding, M., James, A. J., Lin, X., Simeone, A., Leckman, J. F. and Vaccarino, F. M. (1995). Fibroblast growth factor 2 increases *Otx2* expression in precursor cells from mammalian telencephalon. *J. Neurosci.* **15**, 7879-7891.

Rubenstein, J. L. R., Shimamura, K., Martines S., and Puelles L. (1998). Regionalization of the prosencephalic neural plate. *Ann. Rev. Neurosci.* **21**, 445-478.

Ruiz i Altaba, A. (1998). Deconstructing the organizer. *Nature* **391**, 748-749.

Shimamura, K. and Rubenstein, J. L. R. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.

Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A. and Wagner, E. F. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J*. **17**, 719-731.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M. R., Nigro, V. and Boncinelli, E. (1993). A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates the anterior neuroectoderm of the gastrulating mouse embryo. *EMBO J*. **12**, 2735-2747.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. and Boncinelli, E. (1992). Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J*. **11**, 2541-2550.

Stemple, D. and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-985.

Stemple, D. L. and Mahanthappa, N. K. (1997). Neural stem cells are blasting off. *Neuron* **18**, 1-4.

Stevanovic, M., Zuffardi, O., Collignon, J., Lovell-Badge, R. and Goodfellow, P. N. (1994). The cDNA sequence and chromosomal location of the human SOX2 gene. *Mammalian Genome* **5**, 640-642.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L, Rijli, F. M., Chambon, P. and Krumlauf, R. (1998). Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Studer, M., Popperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265**, 1728-1732.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274**, 1115-1122.

Temple, S. and Alvarez-Buylla, A. (1999). Stem cells in the adult central nervous system. *Curr. Op. Neurobiol*. **9**, 135-141.

Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C., Barnard, J. A., Yuspa, S. H., Coffey, R. J. and Magnusson, T. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science.* **269**, 230-234.

Tropepe, V., Sibilia, M., Ciruna, B. G., Rossant, J., Wagner, E. F. and van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol*. **208**, 166-188.

Uchikawa, M., Kamachi, Y. and Kondoh, H. (1999). Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonioorganogenesis of the chicken. *Mech. Dev.* **84**, 103-120.

Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J. and Sharpe, P. T. (1995). Embryonic expression of the chicken *Sox2*, *Sox3* and *Sox11* genes suggests an interactive role in neuronal development. *Mech. Dev*. **49**, 23-36.

Vescovi, A. L., Reynolds, B. A., Fraser, D. D. and Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951-966.

Walters, M. C., Magis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groundine, M. and Martin, D. I. (1996). Trascriptional enhancers act in cis to suppress position-effect variegation. *Genes Dev*. **10**, 185-195.

Wood, H. B. and Episkopou, V. (1999). Comparative expression of the mouse *Sox1*, *Sox2* and *Sox3* genes from pre-gastrulation to early somite stages. *Mech. Dev.* **86**, 197-201.

Xuan, S., Baptista, C. A., Balas, G., Tao, W. Soares, V. C. and Lai, E. (1995). Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14**, 1141-1152.

Yamaguchi, T. P., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). fgfr1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev*. **8**, 3032-3044.