

Novel transcription factor *Satb2* interacts with matrix attachment region DNA elements in a tissue-specific manner and demonstrates cell-type-dependent expression in the developing mouse CNS

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Abstract

Satb1 is a first cell-type-specific transcription factor of a novel type that functions as a regulator of the transcription of large chromatin domains. We identified a close homologue of *Satb1*, *Satb2*, in a cDNA subtraction screening in a search for genes controlling neural differentiation. *Satb2* showed 61% amino-acid homology to *Satb1*. *Satb2* and *Satb1* expression was detected in different cell subpopulations of developing mouse CNS in a mutually exclusive manner. In the electrophoretic mobility shift assay we demonstrate that nuclear extracts from the embryonic day 18.5 mouse developing neocortex, in contrast to basal ganglia, contain a protein complex interacting with matrix attachment region DNA elements (MARs) with high affinity. Endogenous *Satb2* protein is a part of this complex. In the developing neocortex *Satb2* was detected largely in the superficial layers. In the developing spinal cord *Satb2* expression marks a subpopulation of *Lbx1*-positive neurons dorsally and a subgroup of *Isl1*-positive neurons ventrally. In the *Lbx1* mutants *Satb2* expression is greatly reduced. We suggest that *Satb2* may regulate differentiation of subsets of neurons at the level of higher order chromatin structure via binding to MARs.

Introduction

The tissue-specific regulation of gene expression is critical to the development and function of all higher eukaryotic organisms. Tissue-specific transcription is controlled by the presence of certain sets of transcription factors that activate or suppress the expression of a subset of genes in a given cell type. In addition, chromatin modification is involved in the regulation of gene activity (Fry & Peterson, 2001). Recent studies suggest the importance of high-order chromatin structure in the regulation of gene expression (Schubeler *et al.*, 2000). Cell-type-specific gene regulation, at the level of chromatin organization, is an important aspect of cellular differentiation. However, this remains poorly studied.

Recently, the first transcription factor of a novel type that seems to be involved in the regulation of tissue-specific organization of chromatin, *Satb1*, was identified. *Satb1* is a nuclear protein that can bind to a core unwinding element within the matrix attachment DNA region (MAR) (Dickinson *et al.*, 1992). Unlike classic transcription factors, which bind individual target genes to regulate transcription, *Satb1*, which binds to multiple sites where chromatin is fastened to form loop domains, may dictate the organization and structure of chromatin domains, thereby orchestrating the transcription potential of multiple genes. *Satb1* ablation led to gene dysregulation affecting multiple genes in T-cells (Alvarez *et al.*, 2000). Gene knock-out studies have also demonstrated that *Satb1* acts as a global regulator of thymocyte

differentiation that provides sites for tissue-specific organization of DNA sequences and regulates region-specific histone modifications (Yasui *et al.*, 2002; Cai *et al.*, 2003). The closest homologue of *Satb1*, *Satb2*, has recently been identified as a gene mutated in human patients with cleft palate (FitzPatrick *et al.*, 2003). It has also been demonstrated that *SATB2* can enhance gene expression and that the protein can be modified by sumoylation (Dobrev *et al.*, 2003).

In this study we characterized the expression of both *Satb1* and *Satb2* in the developing mouse CNS. *Satb2* expression is largely confined to subsets of postmitotic cells in the developing CNS with the highest expression in the developing cerebral cortex. *Satb1* is also expressed in several regions of developing CNS, but their expression patterns do not overlap during development. We have also demonstrated that developing neocortex, but not basal ganglia, contains a nuclear protein complex that can bind nuclear MARs. *Satb2* protein is a part of the complex. Our findings suggest that *Satb2* and *Satb1* represent a novel class of transcription regulators that control neuronal differentiation of distinct neuronal subtypes by targeting chromatin and regulating expression of multiple loci in a manner similar to *Satb1* action within the T-cell lineage.

Materials and methods

MAR DNA labelling

PCR labelling of MAR probes was performed as described by Nikolaev *et al.* (1996). Probes for binding with nuclear extract were prepared as follows: MAR-containing fragments were PCR-amplified

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in a 50- μ L reaction volume using the corresponding plasmid as template and a library primer 5'-ACTGAGCTCGAGTATCCATGACA.

The PCR labelling was conducted in a final volume of 50 μ L containing \sim 10 ng of the template fragment, 20 μ M library primer for MAR fragments, 200 μ M each dCTP, dGTP and dTTP, 20 μ M dATP, 50 μ Ci [α - 32 P]dATP (Obninsk, Russia), 3 mM MgCl₂, 10 mM Tris/HCl (pH 8.3) and 50 mM KCl. The reaction was proceeded as follows: 94 °C for 0.8 min, 50 °C for 0.6 min, 72 °C for 1 min, for 18 cycles. Reaction products were extracted with phenol-chloroform, precipitated with ethanol and dissolved in TE buffer (10 mM Tris/HCl; 1 mM EDTA).

Animals

Wild-type and *lhx1* knock-out mouse embryos were used to isolate tissue samples. All animal manipulations were carried out in accordance with German law and were approved by the Bezirksregierung Braunschweig

Nuclear matrix preparation and in vitro binding of MARs

Animals were killed by cervical dislocation. Cortical hemispheres and basal ganglia of embryonic day (E)18.5 embryos were surgically removed and used for nuclear extract isolation. Nuclear extracts were prepared from embryonic tissue by a high-salt extraction method

(Cockerill & Garrard 1986). Binding of radioactively labelled MARs to the nuclear extract *in vitro* was carried out as described by Izaurralde *et al.* (1988).

Generation of antibodies against Satb2 protein

In order to produce antibody against Satb2 we generated two different peptides based on predicted protein sequence:

1. C-SSPSSSRTPQAKTSTPTT (453–470 amino acids)
2. C-QQSQPTKESSPPREEA (585–600 amino acids).

These peptides were coupled to BSA (Sigma, St Louis, MO, USA) via MBS (Pierce, Rockford, IL, USA). Coupled peptides were injected into rabbits for immunization. The serum was purified using affinity resin columns (Pierce) pretreated with peptides 1 or 2. Purified serum was tested at different dilutions on cryosections in immunohistochemistry experiments and on Western blots with protein isolated from the cortical tissue. Both antibodies (Ab1 and Ab2) gave positive signals with cortical protein extract on Western blots (see Fig. 2). Western blot analysis of the protein extracts from bacterial cells expressing Satb2 cDNA also produced a band of the expected size (data not shown).

RT-PCR amplification of Satb2 cDNA fragment

The cDNA fragment of the predicted protein KIA1034 (Satb2) was amplified with following primers:

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SATB1 : MDHLNEATQGEKHESEMSNNVSDPKGPP . AKIARLEQNGSPLG . RGRLGSTGGKMQGVPLKHSGLMKTNLRRKGTMLPVF
SATB2 : -ERRS-SPCLRDSPPDRRSGSP-V-----PV-V-----M-A---PN-----AVA-AV-----L-----

      80          *          100          *          120          *          140          *
SATB1 : CVVEHYENAI EYDCKEEHAEFVLVRKDMLFNQLIEMALLSLGYSHSSAAQAGLIQVQKWNVPLSYVTDAPDATVAD
SATB2 : ----QLDGS----N-----S----T--A-----Q--K-----

      160          *          180          *          200          *          220          *
SATB1 : MLQDVYHVVT LKIQHSCPKLEDLPPEQWSHTTVRNALKDLLKDMNQSSLAKECPLSQSMISSIVNSTYYANVSAAKC
SATB2 : -----Q--S-----A--N-A-----E--E-----T--

      240          *          260          *          280          *          300          *
SATB1 : QEFGRWYKHF KKTDMVEMDSLS ---ELSQGANHVNFQGPVPGNTAEQPPSPAQLSHGS--QPSVRTPLPNLHPG
SATB2 : -----K--I-VER--REN--DYCV-G-RPMHLP-MN-LASL-K-N--S-.HS-IH-S-PIRNQ-PALQ-IMS--

      320          *          340          *          360          *          380          *
SATB1 : LVSTPISPQLVNQQLVMAQLLNQYAVNRLLAQQS . . LNQQYLNHPPVSRSMNKPLEQQVSTNT EVVSSEIYQWVRD
SATB2 : -L-PQL-----R--IA--H-I--I--S-----H-HPQAI--F-----IP-AVKP . . PTN-SV . . -PD--Q--

      400          *          420          *          440          *          460
SATB1 : ELKRAGISQAVFARVAFNRTOGLLSEILRKEEDPKTASQSLVNLRAMQNFLQLPEAERDRIYQDERERSLNAASAMG
SATB2 : -----SV-----R-----N--V-----M-PNVS-V

      *          480          *          500          *          520          *          540
SATB1 : PAPLLSTPPSRPPQVKTATLATERNGKPENNTM INNASIYDEIQQEMKRAKVSQALFAKVAATKSQGWLCELLRWKED
SATB2 : SSASS-PSS--T--A--S-PT-DLPI-VDGANV--T-A-----N-----N

      *          560          *          580          *          600          *          620
SATB1 : ENRTLWENLSMIRRFSLPQPERDAIYEQESNAVHHGDRPPIIHVPAEQIQQQQQQQQQQQPPPPPPPPPPQPQ
SATB2 : -----CT-----N--H--V--E--.R--SE-MQ-VVQL-P-PV-VLHR--S-PTKES--REE--AP-

      *          640          *          660          *          680          *          700
SATB1 : QPQPQAGPRLPPRQPTVASSAESDENR QKTRPRTKISVEALGILQSFIQDVGLYPDEEAIQTLSQLDLPKYTI IKF
SATB2 : P-P-----TEDSCAK-P-S-----L-----H-----Q--H-----H-----

      720          *          740          *          760          *          770
SATB1 : FQNQRYYLKHGKLGKNSGLEVDVAEYKDEELLKDLLESVDQKNANTLFSVKLEELSVEGSTDVNADLKD . :764
SATB2 : -----HV-----EHL-SA-----TES--NDSEEGSEEMYK-EA---.NADK-KAAP-ETDQR : 733

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FIG. 1. Comparison of protein sequences for mouse Satb1 and Satb2. The numbers refer to the positions of amino acids. The black and open boxes denote the conserved 'Cut' and 'homeo'- domains. The dashes indicate identical amino acids. Dots indicate parts of one protein that do not have analogous amino acids in the other protein.

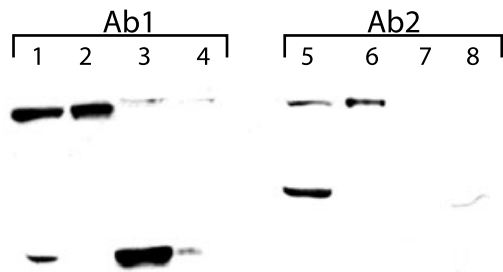


FIG. 2. Western blots with Ab1 and Ab2 anti-Satb2 antibodies. Lanes 1 and 5 contain total proteins and 2 and 6 nuclear extracts isolated from the E18.5 neocortex. Lanes 3 and 4 and 7 and 8 contain nuclear extracts isolated from the HeLa cells and E18.5 basal ganglia, respectively. Note that for each antibody there is a band of size close to the predicted size of 95 kDa and an additional smaller band. The smaller bands, however, are not present in the nuclear extracts (lanes 2 and 6).

Satb2-dir 5'-TTATTGTCAGAGATACTGCGT (exon 7)
 Satb2-rev 5'-TTCTGTTGGAGGAGCGGTG (exon 10)
 The cortical cDNA from E15.5 mice was used as a template. The PCR product was cloned into the pGEM-T cloning system (Promega, Madison, WI, USA). For full cDNA amplification the following primers were designed:

Satb2-ATG 5'-ATGGAGCGGCGGAGCGAGAGC
 Satb2-stop 5'-TTATCTCTGGTCCGTTTCGG.

Immunohistochemistry

Embryos were sectioned at 7–10 μm with a cryostat (Leica), air dried for 20 min, washed in PBS and fixed for 5 min in 4% paraformaldehyde (PFA)/PBS. After three washes in PBS, sections were preblocked in 1% BSA/0.1% Tween 20/PBS (1 h), and incubated with primary antibodies overnight at 4 °C in the same solution. Sections were then washed in PBS and incubated with a diluted (1 : 1000) goat anti-rabbit secondary antibody (Molecular Probes) for 1 h at room temperature, rinsed with PBS and visualized under a fluorescence microscope. The working dilutions of the Satb2 antibodies were 1 : 100 for immunohistochemistry and 1 : 1000 for Western blotting.

The following antibodies were used: guinea-pig anti-Lbx1 (kindly provided by Dr C. Birchmayer), mAb Isl1/2 and Lim1/2 (4F2) (Developmental Studies Hybridoma Bank, University of Iowa, USA), guinea-pig anti-Lmx1b (kindly provided by Dr T. Jessell) and rabbit anti-Tbr1 (kindly provided Dr M. Sheng).

For BrdU birth-dating experiments, BrdU (100 mg/g body weight) (Sigma)/PBS was injected intraperitoneally at E11.5. Incorporated BrdU was detected with mouse anti-BrdU mAb (Sigma).

In situ hybridization

Radioactive *in situ* hybridization was performed as described by Stoykova & Gruss (1994). As probes, coding and non-coding regions

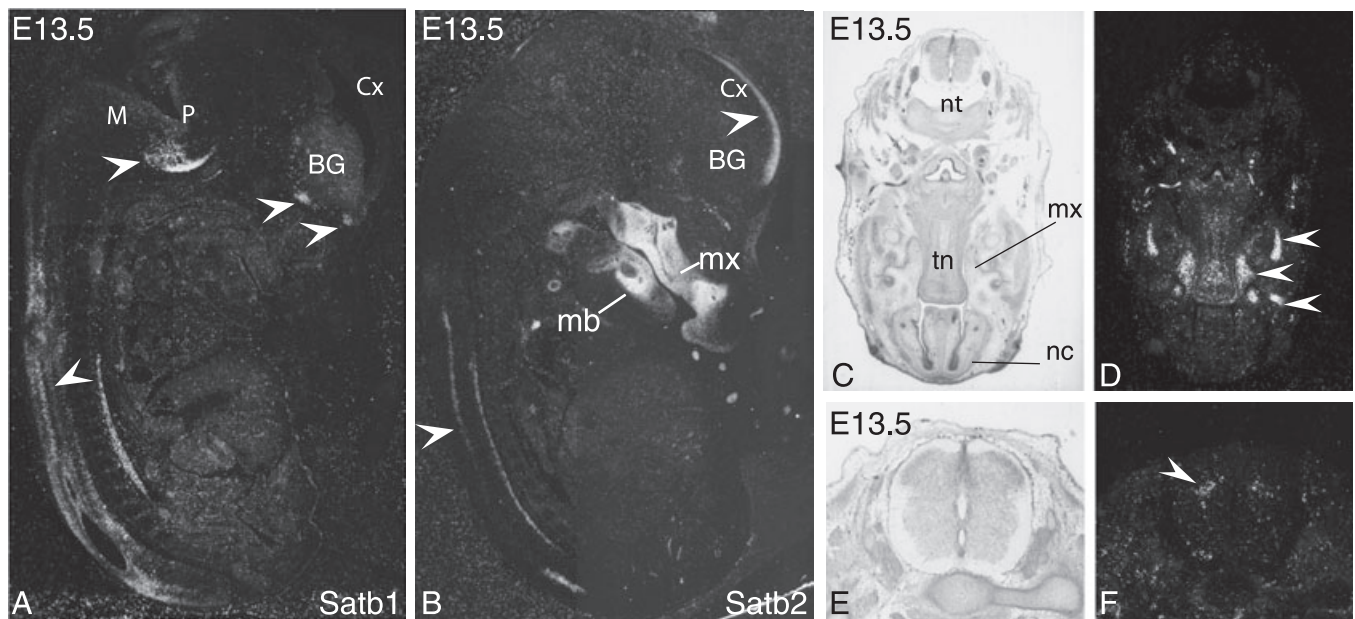
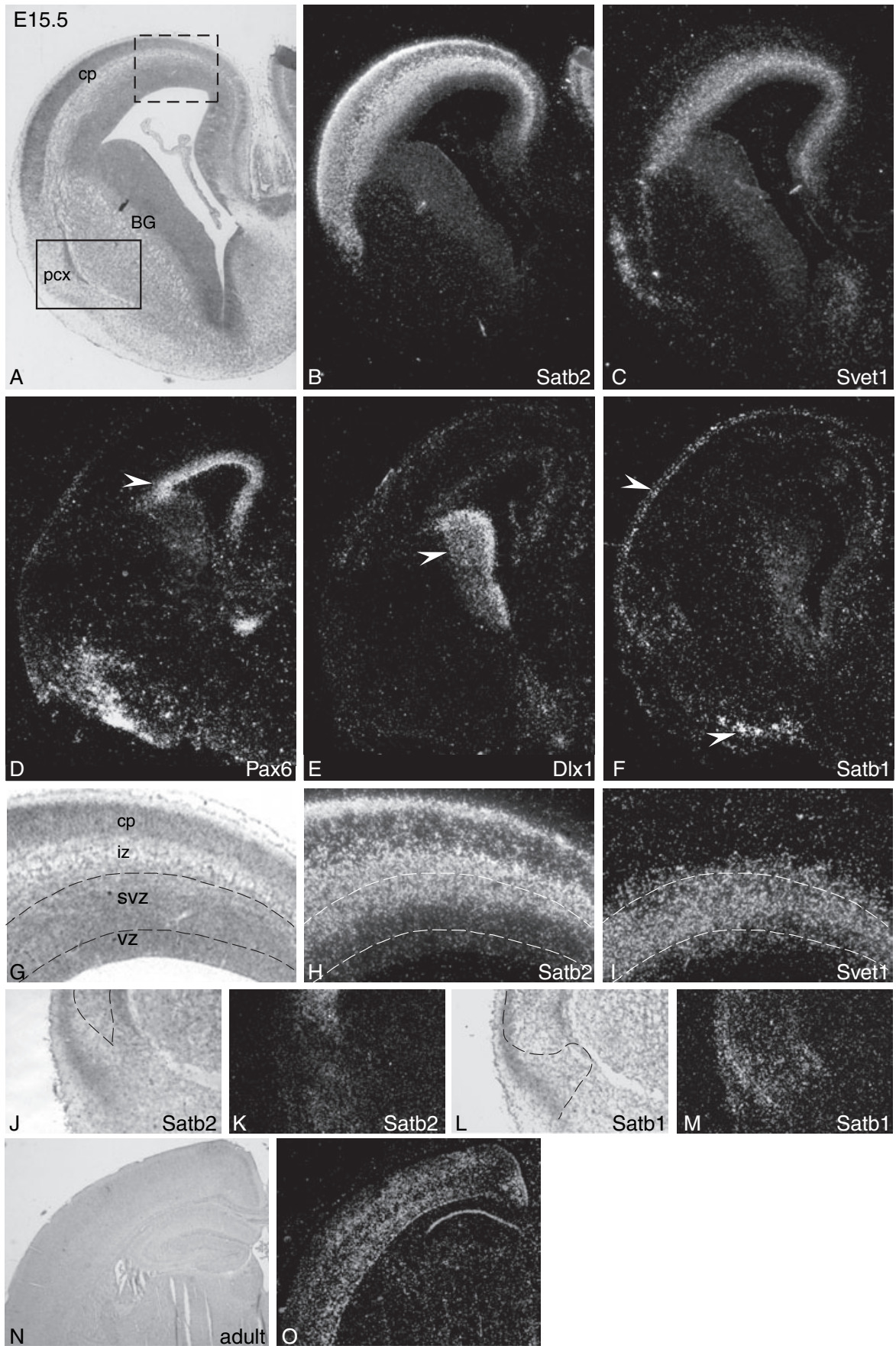


FIG. 3. Satb2 and Satb1 mRNA distribution in the developing mouse embryo at E13.5. (A, B, D and F) Dark-field images; (C and E) bright-field images. At E13.5 *Satb2* and *Satb1* are expressed in a mutually exclusive manner (A and B). *Satb2* is mostly expressed in the neocortex and in the maxilla and mandible (B and D) and in the spinal cord (F; for spinal cord expression see Results). *Satb1* is largely expressed in the spinal cord, in the transition field between medulla and pons and in two groups of cells in the ventral telencephalon (A). Main sites of expression are depicted by arrowheads. Abbreviations: BG, basal ganglia; m, medulla; mx, maxillary component; nc, nasal capsule; nt, notochord; p, pons; tn, tongue

FIG. 4. Comparative expression of *Satb2* at E15.5 in the forebrain. (A, G, J and L) Dark field images; (B–F, H, I, K, M and O) bright-field images. (H and I) Comparison of *Satb2* expression (B) with that of *Svet1* (C), *Pax6* (D), *Dlx1* (E) and *Satb1* (F). G–I and J–L are high-magnification views of the boxed regions from A–C. The dashed lines in G, H and I demarcate the limits of the SVZ. The dashed lines in J and L demarcate the limits of *Satb2* and *Satb1* expression in the piriform cortex. (O) *Satb2* mRNA distribution in the adult brain. Abbreviations: BG, basal ganglia; cp, cortical plate; iz, intermediate zone; SVZ, subventricular zone; pex, piriform cortex; VZ, ventricular zone.



were used. The coding region was amplified with primers Satb2-dir and Satb2-rev (see above).

Results

Identification and structural characterization of a novel *Satb1*-related homeobox gene

In order to identify genes involved in the control of mouse neocortical differentiation we performed a subtractive hybridization of mouse E15.5 PCR-amplified neocortical cDNA against that of E13.5 (Rebrikov *et al.*, 2000). From this cDNA subtraction we selected several genes that were up-regulated in the neocortical subventricular zone (SVZ) at E15.5. A novel cDNA of 1.7 kb (clone A11) showed strong expression in the upper part of the cortical SVZ at E15.5 stage (see below).

In order to isolate a full cDNA copy of the corresponding gene we applied a modified 5'-RACE technology (Matz *et al.*, 1999) that allowed us to isolate 9.6 kb of the A11 clone upstream region. This region did not contain any significant open reading frame, indicating that the cloned part of the gene belonged to either the 3'/5' untranslated region (UTR) or was an intronic part of the underspliced RNA captured in the process of cDNA preparation. The latter hypothesis was confirmed after analysis of the Celera database. The isolated 9.6-kb sequence showed 100% homology to an intronic part of a gene *KIA1034* identified in the Kazusa cDNA sequencing project (Kikuno *et al.*, 2000). In order to confirm the expression pattern of *KIA1034*, we amplified a 538-bp part of a coding region from exons 7–10 of *KIA1034* by PCR from the mouse E15.5 neocortical cDNA. This cloned fragment was used as a probe for *in situ* hybridization on paraffin sections. It produced an expression pattern identical to that of clone A11, indicating that A11 was a cDNA product of underspliced *KIA1034* mRNA.

The *KIA1034*-predicted cDNA encoded a 733-amino-acid protein of 85.5 kDa. A GenBank protein homology search revealed that the *Satb1* homeobox-containing gene was its closest homologue. We referred to the gene therefore as *Satb2*. Alignment of the *Satb1* and *Satb2* amino acid sequences showed an identity of 61% and similarity of 73% (Fig. 1). Both proteins have two 'cut' domains and an atypical homeodomain. A search of the GenBank database did not reveal anything other than *Satb1* homologues, indicating that *Satb1* and *Satb2* are the only members of *Satb* subfamily of cut domain homeobox genes in the mammalian genome.

Satb2 antibody production and specificity verification

In order to extend the analysis of the *Satb2* protein two antisera were raised in rabbits. The first, Ab1, was directed towards the peptide localized between amino acids 453 and 470 of the protein and the other, Ab2, was directed towards the peptide localized at positions

585–600 (Fig. 1, see also Materials and methods). Both peptide sequences were selected in the regions of the protein that did not show any homology to *Satb1* and were unique for *Satb2*. In order to test these two antisera, *Satb2* recombinant protein was produced in *Escherichia coli*. Western blots probed with Ab1 or Ab2 strongly revealed the protein (data not shown). Both antibodies were then tested for specificity by Western blotting on homogenates of embryonic neocortex, basal ganglia and HeLa cells (Fig. 2). Two protein bands were recognized by both antibodies. The heavier bands, with an apparent molecular weight of approximately 95 kDa, were identical for both antibodies (the predicted molecular weight of the *Satb2* protein is 85.5 kDa). Two other bands had a size of 30 kDa for Ab1 and 50 kDa for Ab2. These bands did not appear in the cortical nuclear extracts, indicating that the corresponding cross-reacting antigens were present in the cytoplasm but not in the nucleus (Fig. 2).

We also performed immunohistochemical experiments on frozen and paraffin sections of embryos or brains taken from different stages of gestation. Both antibodies produced nuclear staining typical for a transcription factor (see below). The signal was always detected in the tissues where *Satb2* RNA was detected too (see description of *Satb2* protein expression below).

All these results indicate that both antibodies specifically recognize *Satb2* in the nuclear extracts and nuclei in the tissue.

Developmental distribution of *Satb2* mRNA and protein suggests a role in neuronal differentiation

The tissue distribution of *Satb2* RNA and protein was analysed by radioactive *in situ* hybridization and immunohistochemistry (Figs 3–6). We also compared *Satb2* expression with that of *Satb1* and several other molecular markers. The first site of *Satb2* expression was detected at E11.5 within the mandibular and maxillary components of the first developing brachial arch (Fig. 5A and B). At E13.5 *Satb2* mRNA was additionally detected in the telencephalon, tongue muscles and in a subpopulation of dorsal spinal cord neurons (Fig. 3B, D and F). *Satb1* expression at E13.5 was broader than that of *Satb2*. In contrast to *Satb2*, *Satb1* mRNA was detected in the whole spinal cord and in two groups of postmitotic cells of the ventral telencephalon (Fig. 3A). Interestingly, *Satb1* was strongly expressed in the region of the pons/medulla transition (Fig. 3A). *Satb1* signal in the cerebral cortex at E13.5 was mostly confined to the SVZ zone of differentiating cells (Fig. 3B).

At E15.5, within the cortex and SVZ, *Satb2*-positive cells were detected in the intermediate zone (IZ) and cortical plate (CP) (Fig. 4B and H). *Satb1* expression at E15.5 within the telencephalon was detected in the cells of the marginal zone (MZ) and subplate cells of the developing hippocampus (Fig. 4B and H). *Satb1* was also detected in the piriform cortex and olfactory tubercle (Fig. 4F). Interestingly, medial boundary of *Satb1* expression in the piriform cortex coincided with the lateral boundary of *Satb2* expression in the neocortex

FIG. 5. *Satb2* protein distribution and birth dating of *Satb2*-expressing neurons. (A) Sagittal and (B) transverse sections of E11.5 mouse embryos. *Satb2* protein is expressed in the cells of lateral nasal process and developing jaws. (E) *Satb2* protein distribution in the E13.5 diencephalon. Three groups of *Satb2* cells are depicted by arrowheads. (G) *Satb2* protein in the E15.5 telencephalon. *Satb2* expression in neocortex (C) and spinal cord (D) 24 h after a BrdU pulse. Double-labelled cells are absent from both the telencephalon and the spinal cord. Abbreviations: Aa, amygdala; cp, cortical plate; Hth, hypothalamus; iz, intermediate zone; ln, lateral nasal process; lv, lateral ventricle; ms, mandibular swelling; pex, piriform cortex; sp, spinal cord; Th, thalamus.

FIG. 6. Colocalization of *Satb2* protein with Tbr1, reelin and calbindin in the neocortex. IHC with polyclonal anti-*Satb2* (A) and anti-Tbr1 (B) antibody at E15.5. C and D are high-magnification views of the boxed regions shown in A and B. Double IHC with polyclonal anti-*Satb2* and monoclonal anti-reelin (E) and anti-calbindin (F) antibodies at P0.

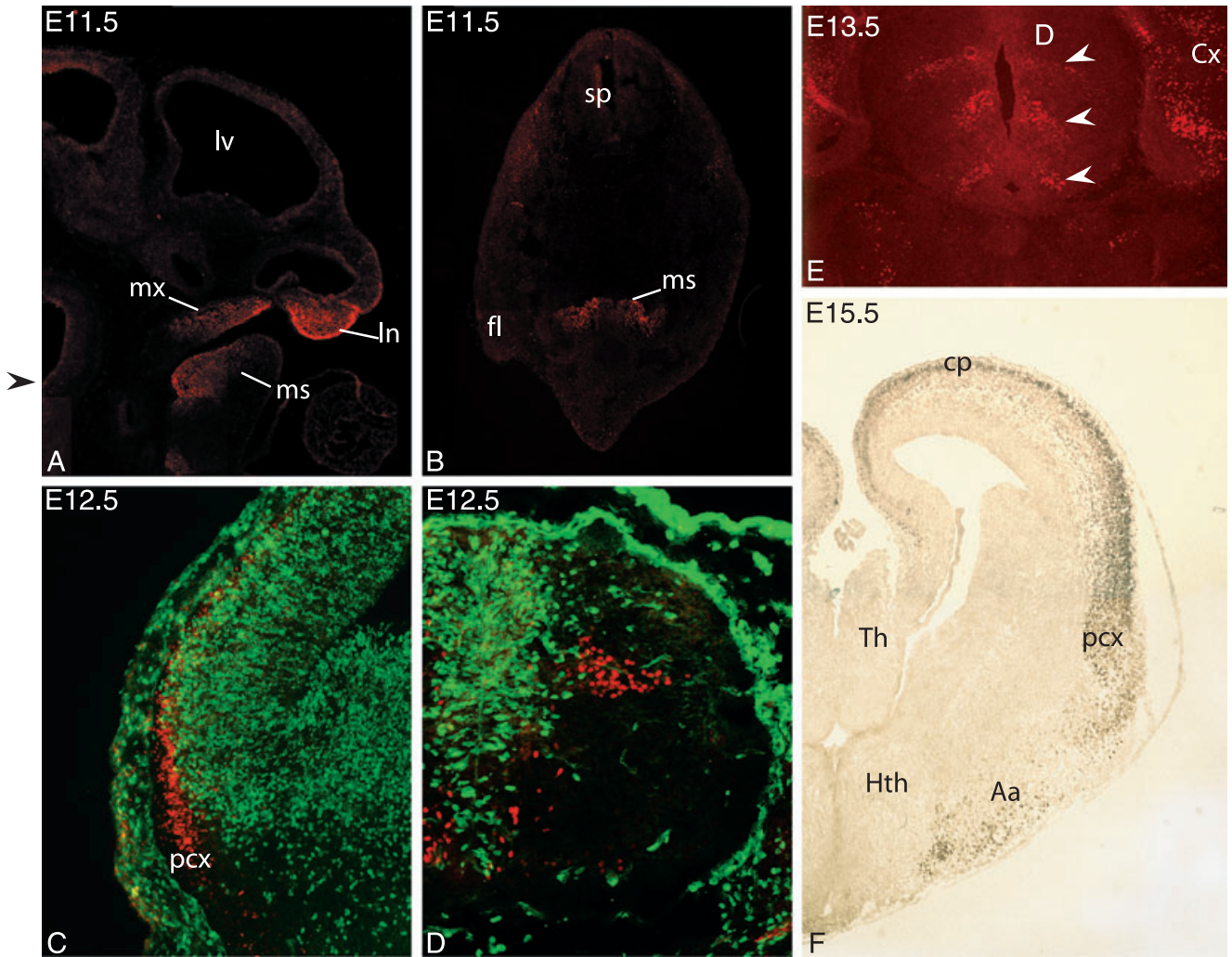


FIG. 5.

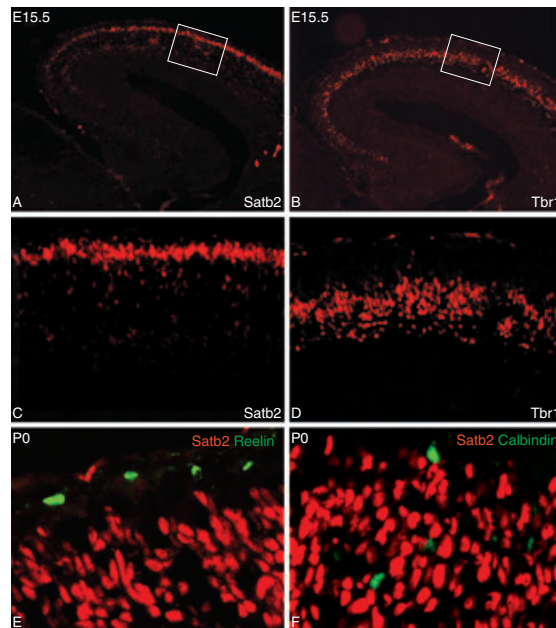


FIG. 6.

(Fig. 4J–M). This boundary also coincided with the anatomical boundary between the neocortex and piriform cortex (Fig. 4J and L). By contrast, the medial boundary of *Satb2* expression coincided with the anatomical boundary between the subiculum and hippocampus (Fig. 4A and B). Expression patterns of *Satb2* and *Satb1* did not overlap in the telencephalon, indicating that they do not have a redundant function in this tissue.

In order to attribute *Satb2* expression to certain subpopulations of the developing telencephalon we compared its expression with the expression of other molecular markers. These markers included reelin as a marker of Cajal-Retzius cells, calbindin as a marker of some GABAergic cells, *Dlx1* as a marker of basal ganglia born interneurons (Anderson *et al.*, 1997), *Tbr1* as a marker of neocortical deep layers (Hevner *et al.*, 2001), *Pax6* as a marker of the pallial ventricular zone (VZ) and *Svet1* as a marker of the SVZ. Expression of *Satb2* neither overlapped with the expression of *Pax6* nor overlapped with the expression of *Dlx1* (Fig. 4B, D and E). While *Pax6* signal in the pallium was largely confined to the VZ, *Satb2* signal was detected in the SVZ, IZ and CP. Expression of *Satb2* in the neocortical SVZ partially overlapped with the expression of *Svet1*. *Svet1* expression labels the whole SVZ at E13–E15 (Tarabykin *et al.*, 2001). In contrast, *Satb2* mRNA within the SVZ was detected largely in its upper parts (Fig. 4J–I). Comparing their expression at latter stages we observed that while *Svet*-positive cells stay in the SVZ until E17.5 (Tarabykin *et al.*, 2001), a significant portion of *Satb2*-positive cells begin migration towards the cortical plate as early as E14.5 (data not shown). At E15.5 many *Satb2*-positive cells have already reached the CP (Fig. 4B and H). By E18.5 the majority of *Satb2*-positive cells occupy the upper part of the CP (data not shown).

Double immunohistochemistry (ICH) with anti-*Satb2* and anti-reelin antibodies at E15.5 and postnatal day (P)0 did not reveal any double positive cells (Fig. 6E). Reelin-positive cells were located in the MZ, which was free from *Satb2*-positive cells. Most of the calbindin-positive cells did not express *Satb2* either (Fig. 6F), although we did detect a few cells weakly labelled by both calbindin and *Satb2* (data not shown). We also compared the expression of *Satb2* and *Tbr1* proteins on adjacent sections at E15.5. Most of the *Satb2*-positive cells were detected in the upper part of the CP whereas *Tbr1*-positive cells occupied the deeper part of the CP (Fig. 6A–D). These data indicate that most of the *Satb2*-positive cells do not coexpress *Tbr1*, although it is possible that there is a minor portion of *Satb2* cells that could also express *Tbr1*.

ICH staining for *Satb2* protein at E15.5 showed that roughly half of the neurons of the CP contained *Satb2* protein (Fig. 5F). *Satb2* protein, in contrast to mRNA, was not detected in the neocortical SVZ cells (Fig. 5F). These results suggest either a posttranscriptional regulation of *Satb2* expression or a lower sensitivity of ICH detection as compared with *in situ* hybridization. Such a difference was not detected for other parts of the CNS expressing *Satb2*. In the developing diencephalon there were three groups of *Satb2*-positive cells: ventrally, medially and dorsally (Fig. 5E). In the adult brain *Satb2* was expressed in the neocortex and hippocampus although its expression is decreased as compared with that in the embryonic brain (Fig. 4O).

Birth-timing of Satb2-positive neurons in the telencephalon and spinal cord

In order to determine the birth-time of *Satb2*-positive neurons we injected pregnant females with BrdU at E11.5. The embryos were taken for IHC analysis 24 h later. Embryo sections were subjected to double IHC with anti-BrdU and anti-*Satb2* antibodies. We did not detect any double positive cells in the neocortex or in the spinal cord (Fig. 5C and D). *Satb2*-positive cells in the telencephalon were located mostly in the lateral cortical plate directly above the layer of BrdU-positive cells (Fig. 5C). A similar situation was observed in the spinal cord – all the *Satb2*-positive cells were situated in the MZ outside the domain of BrdU-positive cells in the VZ (Fig. 5D). These results indicate that *Satb2*-positive cells in the spinal cord and early born *Satb2*-positive cells in the telencephalon are generated before E11.5.

Satb2 expression in the dorsal spinal cord identifies a subgroup of Lbx1-positive neurons and is abolished in lbx1 mutants

The first *Satb2*-expressing cells in the spinal cord were detected at E12.5 and were observed at all axial levels of the spinal cord. Three domains of *Satb2* expression – dorsal, medial and ventral – can be distinguished at this stage within the spinal cord (Fig. 7E). In order to attribute *Satb2* to certain neuronal classes we performed double immunostaining of *Satb2* and other spinal cord neuronal markers. Staining with anti-*Isl1/2* antibody demonstrated that all ventral *Satb2* neurons were also coexpressing *Isl1/2*. However, not all *Isl1/2* ventral neurons were *Satb2* positive (Fig. 7C and E). *Satb2*-positive neurons in the dorsal portion of the spinal cord were not positive for either *Isl1/2*, *Lmx1b* (Fig. 7) or *Evx1* (data not shown). They were, however, positive for both *Lim1/2* and *Lbx1* proteins (Fig. 7). Notably, *Satb2*-positive cells were expressing *Lbx1* at a lower level than most of the other *Lbx1* neurons (Fig. 7G–I). *Satb2*-positive dorsal neurons were located at the lower boundary of dorsal *Lbx1* expression (Fig. 7). Interestingly, a subset of *Satb2*-positive cells located at this stage in the upper portion of the ventral spinal cord do not coexpress any molecular marker tested.

In order to study whether *Satb2* is situated downstream of *Lbx1* transcription factor we studied the expression pattern of *Satb2* in the spinal cord of *lbx1*^{-/-} mutant E12.5 embryos. The amount of *Satb2*-positive cells was significantly decreased in the dorsal part of the spinal cord in the mutants. At the same time, ventral and medial *Satb2* expression domains appeared to remain unchanged (Fig. 8).

Satb2 in the cerebral cortex is a part of a nuclear complex interacting with nuclear MARs

In order to investigate the ability of *Satb2* protein to bind MARs, we first selected those MARs that can interact with nuclear extracts from the tissue with the highest concentration of *Satb2* protein. Developing E18.5 cerebral cortex was selected as a tissue source on the basis of expression experiments. Figure 9 shows an autoradiogram of gel

FIG. 7. *Satb2* expression demarcates a subset of *Lbx1*-positive neurons in the spinal cord. Colocalization of *Satb2* protein with *Lbx1* (A, F), *Isl1/2* (C, E), *Lim1/2* (B) and *Lmx1b* (D) in the E12.5 and E13.5 spinal cord. (G–I) High-magnification views of F. (H) Superimposition of G and I. *Satb2* protein in the dorsal spinal cord is coexpressed with *Lbx1* and *Lim1/2* but not with *Lmx1b* and *Isl1/2*. Arrowheads on G, H and I depict a typical *Lbx1*⁺/*Satb2*⁺ neuron. Level of *Lbx1* expression is lower than in a neighbouring *Lbx1*⁺/*Satb2*⁻ neuron. (J) Schema of updated classification of dorsal spinal cord neurons including *Satb2* as a possible marker of d16 neurons.

FIG. 8. *Satb2* expression is decreased in the spinal cord of *lbx1*^{-/-} mutant. Colocalization of *Satb2* (C) and *Isl1/2* (A) in the E12.5 spinal cords of *lbx1*^{-/-} mutant. (B) Superimposition of A and C. Note the remaining group of *Satb2*-expressing cells in the ventral part of the spinal cord on C. (D) *Satb2* expression in the spinal cord of E12.5 *lbx1*^{-/-} mutant and a wild-type littermate (E). Arrowhead in D indicates the region corresponding to the region of dorsal *Satb2* cells in the wild type.

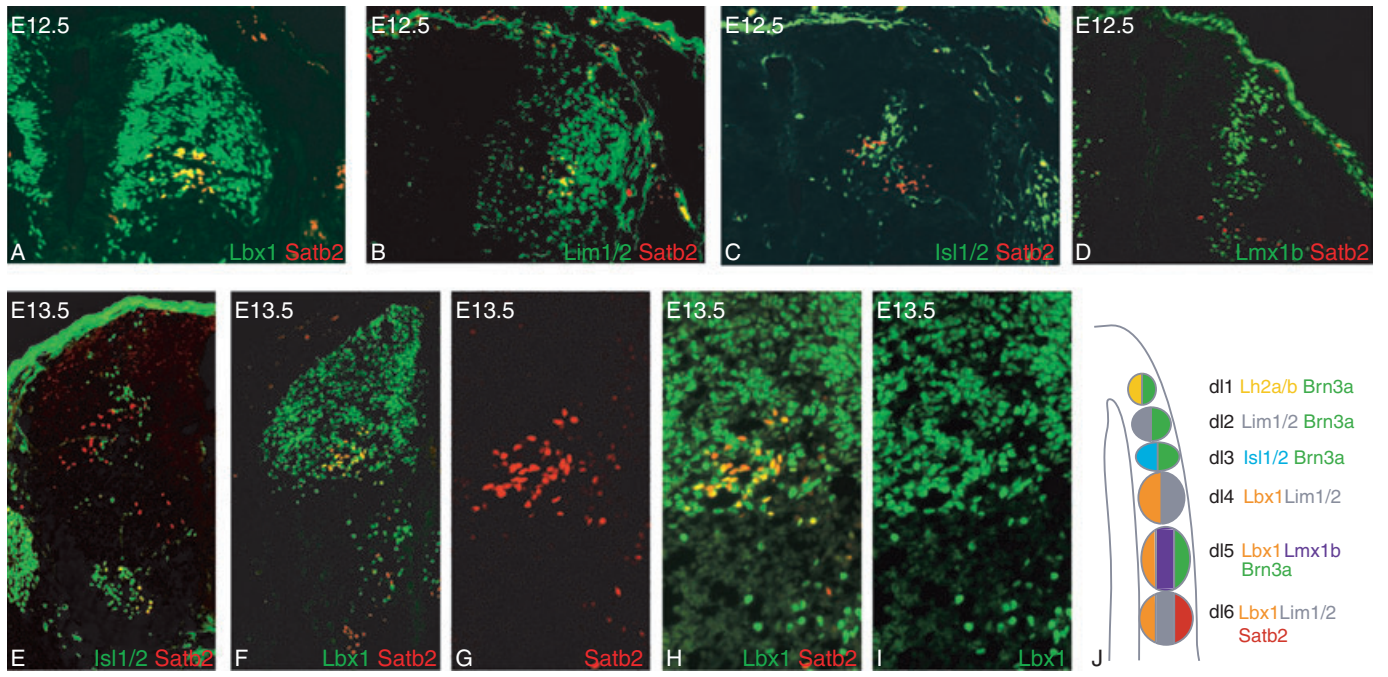


FIG. 7.

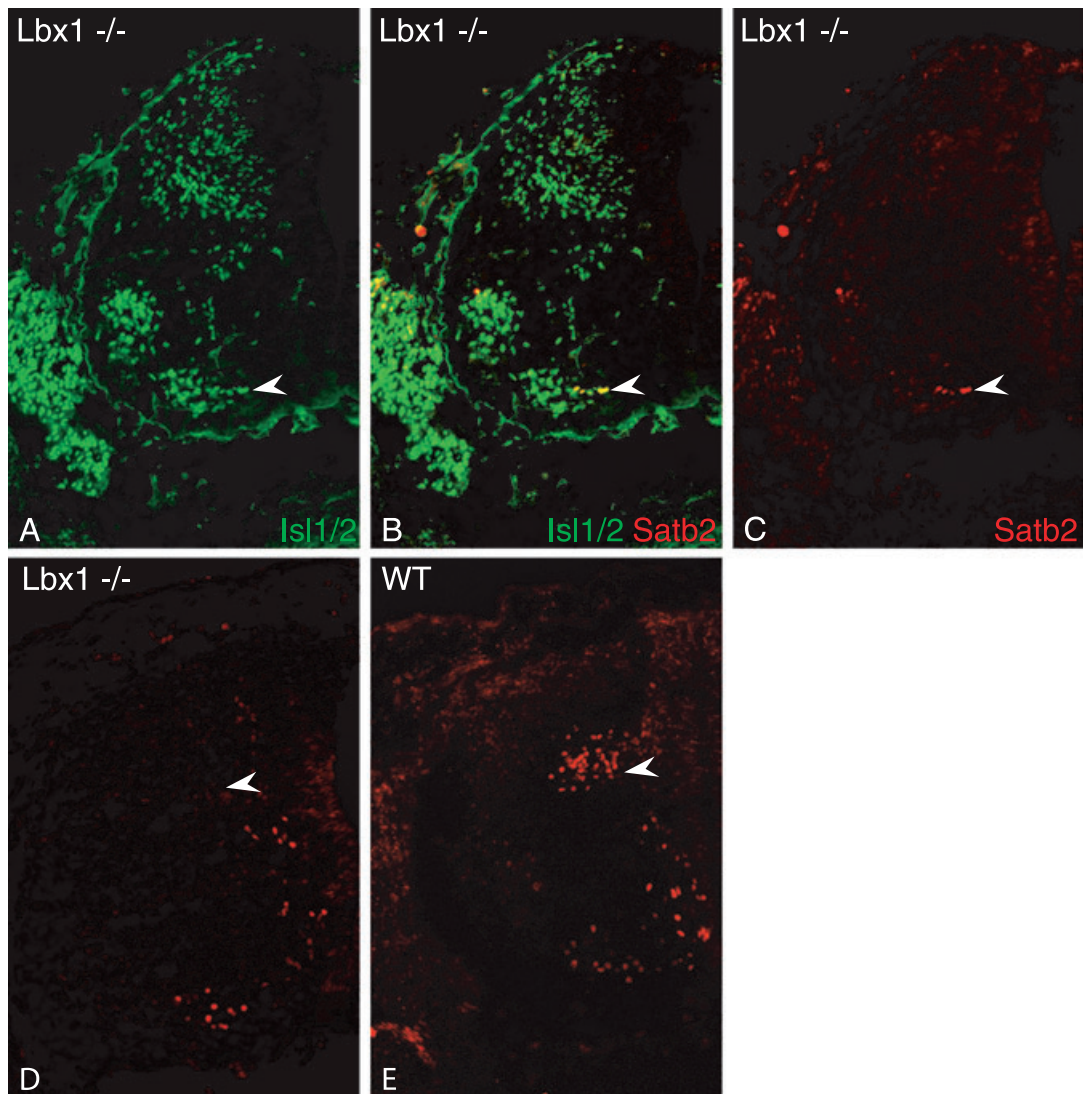


FIG. 8.

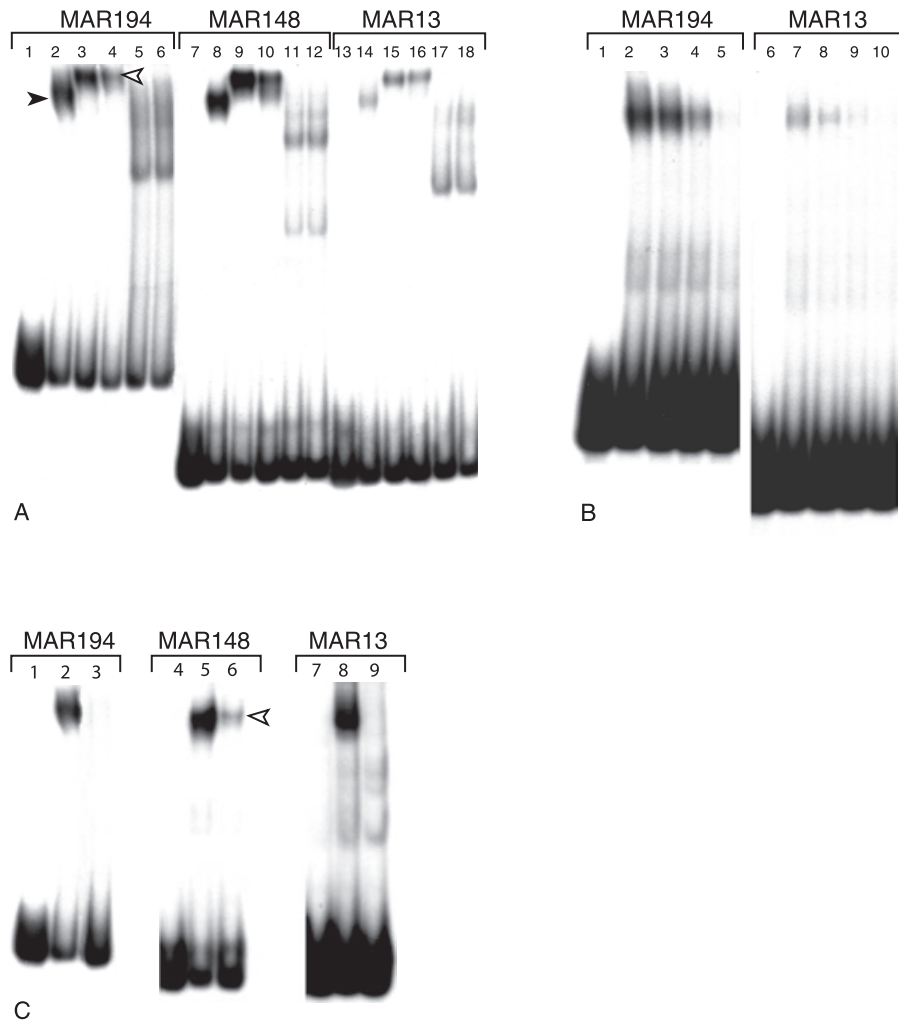


FIG. 9. Interaction of Satb2 containing protein complex with MAR DNA. (A) Gel mobility shift assay with three different radiolabelled MARs. Lanes 1, 7 and 13 contain samples with no protein. Lanes 2, 8 and 14 contain MARs that were reacted with mouse cortical nuclear extracts. Lanes 3, 9 and 15 were loaded with the binding reaction, additionally containing anti-Satb2 antibody Ab1. Lanes 4, 10 and 16 contain the binding reaction containing Ab2 antibody. Lanes 5, 11 and 17 contain negative control samples with nuclear extracts isolated from the HeLa cell line that were reacted with MARs with (lanes 6, 12, 18) or without (lanes 5, 11, 17) Ab1 antibody. 'Band shift' and 'superband shift' are depicted with arrowheads. (B) Competition assay. Lanes 3–5 and 8–10 contain increasing amounts (10, 50 and 200 ng) of specific unlabelled competitor DNA. Lanes 1 and 6 do not contain any protein; lanes 2 and 7 do not contain the competitor DNA. (C) Nuclear extracts from the E18.5 mouse neocortex have stronger MAR binding activity than those from the E18.5 basal ganglia. Lanes 1, 4 and 7 do not contain any protein; lanes 2, 5 and 8 contain samples with nuclear extracts isolated from the neocortex; lanes 3, 6 and 9 contain samples with nuclear extracts isolated from the basal ganglia. Note that all three MARs showed formation of a strong single shifted band in for the neocortex, but only in one case was there formation of a much weaker band for the basal ganglia (arrowhead).

mobility shift experiments carried out with hamster and human MARs. The MARs taken for the analysis have been shown to attach to the nuclear scaffold (Nikolaev *et al.*, 1996). In six of ten MAR sequences tested, the addition of nuclear extracts from the cortical tissue resulted in the appearance of a shifted band (Fig. 9A). The shifted bands showed an appearance of a single major binding complex. Interestingly, nuclear extracts isolated from E18.5 basal ganglia tissue for all but MAR148 did not produce strong shifted bands (Fig. 9C). In the case of MAR148 (M2E4) a weaker band of similar size to that of cortical band. Formation of the shifted complex was inhibited by specific unlabelled competitor DNA (Fig. 9B).

We then tested whether Satb2 is a part of the MAR-binding complex in the cerebral cortex. The presence of Satb2 protein in the cortical nuclear extracts was confirmed on a Western blot (Fig. 2) using Ab1 and Ab2 antibodies. In order to detect Satb2 within the cortical MAR binding complex, Ab1 antibody was added to the binding reaction. For all three MARs tested this resulted in the

supershift of the DNA–protein complex (Fig. 9A). In the negative control experiments the antibody was added to the nuclear extract isolated from HeLa cells, where Satb2 protein was not detected. These experiments, as expected, did not produce any band supershift.

Discussion

Satb2 expression delineates subsets of differentiating neurons in the developing CNS

The problem of characterization of different cell types is a common one in many regions of the CNS. The molecular profile of a certain cell type is an important aspect of neuronal phenotype. In this study we investigated Satb2 mRNA and protein distribution with embryonic development. Most of the cells expressing the gene were found within the CNS and developing cartilage tissue. In all regions of the CNS, Satb2 expression peaked in differentiating neurons and decreased in

mature cells. This suggests that *Satb2* is involved in the control of neuronal differentiation. The highest proportion of *Satb2*-expressing neurons, greater than 50%, was detected in the neocortex between E15.5 and E18.5. In the other parts of the CNS where *Satb2* was expressed, such as spinal cord and thalamus, *Satb2*-positive cells were minor subpopulations comprising not more than 5–10% of all cells. Interestingly, in the neocortex, *Satb2* protein, in contrast to mRNA, was not detected in the SVZ but was detected in the CP. Although this finding could be a result of low sensitivity of IHC, it may also be explained by a *Satb2* translation block in the neocortical SVZ cells.

Novel molecular classification of cortical neurons has been suggested recently by Hevner *et al.* (2003). Cortical neurons were classified on the basis of gene expression profiles. Our data corroborate *Satb2* as a valuable marker of cortical superficial layers. *Satb2*-positive cells do not express reelin, and most do not express *Tbr1* and calbindin. Our data also indicate that *Satb2* is not expressed in the cells that express another marker of superficial layers and the SVZ, *Svet1*. However, there may be minor subgroups of cells coexpressing *Satb2* and *Svet1*, or *Satb2* and *Tbr1*. Further experiments will need to be performed to address this issue.

Recently, a new classification of dorsal spinal cord neurons was suggested (Gross *et al.*, 2002; Muller *et al.*, 2002). According to the new classification early dorsal neurons consist of two major classes, *Lbx1*-negative (class A) neurons and *Lbx1*-positive (class B) neurons. The latter are subdivided into three classes: D3 (dI4), D4 (dI5) and D5 (dI6). These neurons are distinguished on a basis of the expression of certain combinations of transcription factors that control their differentiation. Two of these groups, however, D3 (dI4) and D5 (dI6), have been undistinguishable by means of molecular markers. Both D3 (dI4) and D5 (dI6) neurons coexpress *Lbx1* and *Lim1/2*. Thus far, they have been distinguished only by their positions along the dorso-ventral axis (Gross *et al.*, 2002; Muller *et al.*, 2002). Our colocalization experiments demonstrated that dorsal *Satb2*-expressing cells also express *Lbx1* and *Lim1/2* but do not express *Lmx1b* or *Isl1*. The fact that dorsal *Satb2* neurons are located in the most ventral part of the *Lbx1* expression domain suggests that *Satb2*-expressing cells could be either dI4 or dI6 neurons. This is also supported by the fact that *Satb2*-positive neurons in the spinal cord appear before E11.5.

Additionally, we have detected a decrease of *Satb2*-expressing cells in the dorsal part of the spinal cord of *lhx1*^{-/-} mutants. It has been shown that inactivation of the *Lbx1* gene in the spinal cord leads to ablation of class B neurons (Gross *et al.*, 2002; Muller *et al.*, 2002). It is not clear whether *Satb2* is a direct target of *Lbx1*, or whether the lack of *Satb2*-positive cells in the dorsal part of the spinal cord is the result of re-specification of these cells.

Satb2 and *Satb1* as potential chromatin remodelling regulators in the developing CNS

Using the cDNA subtraction approach we isolated and characterized a novel mouse gene *Satb2* that encodes a *Satb1* homologous transcription factor. We demonstrate that endogenous *Satb2* protein is a part of a protein complex that binds to the MARs. It has been demonstrated that *Satb1* also binds to MARs and regulates expression of multiple genes in the maturing T-cells. It is suggested that *Satb1* acts as a global regulator of thymocyte differentiation that provides sites for tissue-specific organization of DNA sequences and regulates region-specific histone modifications. It has been reported that *Satb1* is expressed predominantly in thymocytes (Dickinson *et al.*, 1992; Adams *et al.*, 1993) but was also detected in the fetal brain (Adams *et al.*, 1993). We demonstrate here that during development *Satb1* is

expressed in several regions of the developing CNS. *Satb2* is also expressed in the developing CNS, but expression patterns of *Satb2* and *Satb1* do not overlap. In the developing cerebral cortex, for example, *Satb1* is expressed in the MZ and piriform cortex whereas *Satb2* is not expressed in these cells. *Satb2*, in turn, is largely expressed in the superficial layers of the neocortex and subiculum. It has been reported that *Satb1* knock-out mice have a neurological phenotype such as incomplete eye opening and the clasping reflex (Alvarez *et al.*, 2000). Our results, together with the previous reports, suggest that *Satb2* and *Satb1* can act as global regulators of cell differentiation in specific cell lineages within the developing CNS. The molecular basis for *Satb1* action was recently demonstrated by Kohwi-Shigematsu and coauthors (Yasui *et al.*, 2002; Cai *et al.*, 2003). They have shown that *Satb1* forms a three-dimensional network structure in the thymocyte nuclei and recruits the histone deacetylase into this network. Their results convincingly demonstrate that *Satb1* defines a novel class of transcription regulators that function as a protein defining the sites within a chromosome that should be subjected to chromatin remodelling. *Satb2* has been recently identified as a gene mutated in human patients with cleft palate. It has also been demonstrated that *Satb2* can enhance gene expression and the protein can be modified by sumoylation (FitzPatrick *et al.*, 2003; Dobrova *et al.*, 2004).

The mutually exclusive expression of *Satb2* and *Satb1* suggest that they control the expression of distinct subsets of genes at the level of chromatin in the developing CNS. Within the CNS there are still cells that do not express either *Satb1* or *Satb2*. Because we did not identify anything other than *Satb1* homologues of *Satb2* in the mouse genome, it is likely that there should be other proteins similar to *Satb1* and *Satb2* functioning in other neural cells. It remains to be elucidated whether *Satb1* and *Satb2* can be substituted for one another or whether each has a unique set of targets. Further experiments involving 'knock-in' experiments need to be performed in order to address this question.

For band-shift analysis we randomly selected ten MARs from the human and hamster genome. Six of these MARs strongly interact with a nuclear protein complex isolated from developing neocortex. We have also demonstrated that endogenous *Satb2* protein is a part of the MAR-interacting complex. Note that none of the MARs demonstrated strong interaction with basal ganglia nuclear proteins isolated from the same stage. This surprising finding suggests that the level of chromatin remodelling reactions at E18.5 is higher in the neocortex than in the basal ganglia. It can be explained by the fact that the basal ganglia tissue, although most closely related to the neocortex, is more developmentally advanced at E18.5 and that most of the chromatin remodelling processes could be completed by E18.5. Another possible explanation is that the neocortex, having a higher cellular diversity in general, requires a higher level of chromatin remodelling. The latter hypothesis is also supported by the fact that a histone acetyltransferase gene *Querkopf* is strongly expressed in the neocortex and is required for normal cerebral cortex development (Thomas *et al.*, 2000).

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Abbreviations

CP, cortical plate; ICH, immunohistochemistry; IZ, intermediate zone; MAR, matrix attachment region DNA element; MZ, marginal zone; SVZ, subventricular zone; VZ, ventricular zone.

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