MOLECULAR AND CELLULAR BIOLOGY, Dec. 2003, p. 9046–9060 Vol. 23, No. 24
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.24.9046–9060.2003


# Postnatal Lethality in Mice Lacking the *Sax2* Homeobox Gene Homologous to *Drosophila S59/slouch*: Evidence for Positive and Negative Autoregulation

Ruth Simon and Thomas Lufkin*

*Brookdale Center for Developmental and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029-6574*

Received 2 July 2003/Returned for modification 20 August 2003/Accepted 18 September 2003

**Homeobox gene transcription factors direct multiple functions during development. They are involved in early patterning of the embryo as well as cell specification, cell differentiation, and organogenesis. Here we describe a previously uncharacterized murine homeobox gene, *Sax2*, that shows high similarity to the *Drosophila S59/slouch* and murine *Sax1* genes. We show that *Sax2* gene expression occurs early during embryogenesis in the midbrain, the midbrain-hindbrain boundary, the ventral neural tube, the developing eye, and the apical ectodermal ridge of the limb. To determine the role of *Sax2* during development, we generated a knockout mouse line by replacing part of the *Sax2* coding sequences with the *lacZ* gene. The *Sax2* null allele mutants exhibit a strong phenotype indicated by growth retardation starting immediately after birth and leading to premature death within the first 3 weeks postnatal. Intriguingly, our studies also demonstrated a striking autoregulation of the *Sax2* gene in both positive- and negative-feedback mechanisms depending on the specific cell type expressing *Sax2*.**

Homeobox genes play crucial roles during development as has been most extensively described for the *Hox* genes in *Drosophila*. In addition to the *Hox* genes, there are more than 20 homeobox gene families, most of which are involved in the development or cell type specification within the developing embryo. Homeobox genes have an established role in governing the development of the nervous system, and early during embryogenesis homeobox genes are involved in patterning of the future brain and the spinal cord. While the *Hox* genes are expressed in the spinal cord and the hindbrain with a rostral border of expression in rhombomere 2, members of other homeobox gene families (e.g., *Dlx*, *Emx*, *Otp*, and *Otx*) are involved in directing the development of the rostral brain. Early on, the brain is divided into the forebrain, midbrain, and hindbrain regions, and these regions undergo further subdivisions throughout development. Recently much attention has been focused on the midbrain-hindbrain boundary region, the isthmic organizer, one of the two major organizers responsible for the development of the brain. It has been shown that several homeobox genes are involved in establishing the isthmic organizer. Antagonistic expression of the homeobox genes *Otx2* and *Gbx2* defines the precise position of the isthmic organizer, while other homeobox genes such as *Pax2/5* and *En1/2* are required for the isthmic organizer activity (reviewed in references 43 and 54). Homeobox genes also play an important role in the developing neural tube, especially in the patterning and cell fate specification of the ventral neural tube. The dorsal-ventral patterning of the ventral neural tube is dependent on inductive signals derived from the floor plate and the notochord, mediated largely by the secreted protein *sonic hedgehog* (*Shh*). *Shh* induces the differentiation of five distinct neural subtypes at defined positions in the ventral neural tube that are identified by the combinatorial expression of a group of homeodomain proteins. These homeodomain proteins can be divided into two classes by their pattern of regulation: class I homeobox genes are repressed and class II homeobox genes are activated by *Shh*. Pairs of class I and class II homeodomain proteins are expressed in mutually exclusive progenitor domains defining the boundaries of these domains, and their cross-repressive interaction defines the neuronal subtype identity (reviewed in references 5, 27, and 38).

The homeobox gene *Sax2* and its predicted translation product show high similarity to the *Drosophila S59/slouch* gene and the mouse *Sax1* gene (11, 44, 45). Aside from mouse and *Drosophila*, members of this family have been described in chicken (41), *Caenorhabditis elegans* (19), flatworms (35), *Xenopus* (26), and humans (32). In mammals there appear to be only two *Sax* genes forming the *NK1* gene family, which is part of the *NKL* gene cluster (20). Other gene families of this cluster include the *Hmx*, *Msx*, and other *NK* gene families. Expression patterns for the *NK1* gene family have been described in *Drosophila*, mouse, and chicken. *Drosophila S59/slouch* gene expression occurs about 5 h after fertilization in mesoderm cells that develop into muscle founder cells. In addition, the *Drosophila S59/slouch* gene is expressed in the developing nervous system and to a lesser extent in the midgut (11). The mouse *Sax1* gene is expressed early in the ectoderm lateral to the primitive streak, and later *Sax1* expression is restricted to distinct areas of the spinal cord; it is especially high in rhombomere 1 and in the pretectum (45). It has been shown that *Sax1* mRNA is most abundant in day 10 embryos and gradually decreases in day 12 and 14 embryos (44). The expression pattern of *Sax1* suggests two different functions for

* Corresponding author. Mailing address: Brookdale Center for Developmental and Molecular Biology, Mount Sinai School of Medicine, Box 1020, One Gustave L. Levy Place, New York, NY 10029-6574. Phone: (212) 241-5359. Fax: (212) 214-0873. E-mail: thomas.lufkin@mssm.edu.

the gene during development; an early involvement in the determination of the posterior neuroectoderm and a later involvement in the specification of specific subsets of neurons (45). The similarities in the expression pattern and the amino acid sequence among the *Drosophila S59/slouch* and the mouse *Sax1* genes and the *Sax2* gene led us to speculate that *Sax2* may also play an important role in the development of the nervous system. We used RNA in situ hybridization on paraffin sections to determine the expression pattern for the *Sax2* gene during embryogenesis. Here we show that the onset of *Sax2* gene expression occurs as early as embryonic stage E10.5 in the ventral central nervous system and overlaps with the expression pattern of the *Sax1* gene. To further investigate the role of *Sax2* during development, we generated a *Sax2* knockout mouse line by inserting the *lacZ* gene into the coding sequence and analyzed the expression pattern in the *Sax2-lacZ* null allele. β-Galactosidase staining, like the RNA in situ hybridization results, revealed the expression of *Sax2* in the limb, the eye, the ventral neural tube, and different specific subsets of cells in the developing rostral brain during embryogenesis.

## MATERIALS AND METHODS

**Generation of a loss-of-function allele for *Sax2* with *lacZ*.** Genomic cloning of *Sax2* was previously reported (7). Plasmid p1083 was generated by cloning an 18-kb *Not*I fragment into the *Sal*I site of vector pTZ18R. An 11.5-kb *Xho*I-*Sal*I fragment containing the *Sax2* gene was subcloned into the *Xho*I-*Sal*I sites of the Bluescript KS vector (pRS1). *Bsp*EI sites located in the predicted second exon and the second intron were used to create the targeting construct. The construct pRS1 was linearized with *Bsp*EI, and the oligonucleotide 5′-CCGGGTACGTA GGAATTCCATATGC was inserted to modify the *Bsp*EI sites and to add *Sna*BI and *Nde*I sites. A *Xho*I-*Sal*I fragment containing the *IRESlacZ/floxedneo* cassette (kindly provided by Carla Tribioli) was treated with Klenow enzyme to create blunt ends. This fragment was inserted into the *Sna*BI site of pRS1, resulting in construct pRS14. The right orientation of the insert was determined by DNA sequencing.

**ES cell culture and chimeric mouse production.** To generate targeted gene disruption in embryonic stem (ES) cells, 10 μg of DNA of pRS14 was linearized by *Ase*I and electroporated into $10^7$ cells as described previously (53). Positive clones were selected by growing the ES cells in the presence of G418 at concentrations in the range of 150 to 450 μg/ml. Altogether, 192 G418-resistant clones were selected and analyzed by Southern blotting using a 5′ external probe (*Spe*I-*Xho*I [see Fig. 2B]), resulting in a 6.6-kb band for the wild-type clone and an 8.8-kb band for the mutant clone. Positive ES cell lines were microinjected into C57BL/6J blastocysts, and the resulting male chimeras were backcrossed to C57BL/J6 females for a mixed genetic background. For β-galactosidase staining, embryos were collected at stages E8.5 to E14.5 as previously described (14).

**cDNA cloning.** mRNA was isolated from embryos at stage E11.5 (Qiagen), and reverse transcription-PCR (Expanding Long Template PCR System; Roche) reactions were performed using specific oligonucleotides corresponding to sequences just downstream of the homeobox (5′AGGCGCTGACACCAGCGCG CCG) and 325 bp downstream of the predicted start codon (5′TCCTGGGGC GGAGCGGGCAGGGCGG). Using these oligonucleotides, a partial cDNA was obtained and subcloned into the pT-Adv vector (AdvanTage cloning kit; Clontech).

**RNA in situ hybridization.** Sagittal, transverse, and coronal sections of wild-type and *Sax2* null allele mutant embryos at stages E10.5 to E18.5 were prepared as previously described (51, 53). Embryo tissues were fixed in 4% paraformaldehyde overnight, washed in phosphate-buffered saline, dehydrated through graded ethanol, given by two changes of Americlear (Fisher), and embedded in Paraplast (Fisher) overnight under vacuum. Sections of 7 μm were cut and floated onto Plus+ slides (Fisher), dried, and stored at 4°C. As a probe, construct p1083 was linearized with the restriction enzyme *Sfi*I and antisense RNA was synthesized in the presence of 35S UTP using T7 RNA polymerase for in situ hybridization on sections of wild-type embryos. Autoradiography was performed by dipping the slides in a 1:3 ratio of $H_2O$:Kodak NBT2 emulsion, air drying, and exposing for 3 to 7 days. Slides were developed in Kodak D19 and counterstained with hematoxylin. For RNA in situ hybridization experiments comparing wild-type and *Sax2* null allele mutants, we used the partial cDNA (RS19) and construct RS37. Construct RS37 consists of a 2.8-kb *Nsi*I fragment, containing sequences starting 1 kb upstream of the first exon to the second intron, subcloned into Bluescript KS vector. RS19 and RS37 were linearized with *Bam*HI and *Eco*RI, respectively, and RNA was synthesized using T7 RNA polymerase.

## RESULTS

**Determination of the *Sax2* expression pattern during embryogenesis.** The *Drosophila S59/slouch* gene and the murine *Sax1* gene are expressed early during embryogenesis (11, 45). Owing to the high similarity between these genes and the *Sax2* gene, we predicted the possibility of an evolutionarily conserved expression pattern. To determine the spatial-temporal pattern of *Sax2* gene expression during embryogenesis, we performed RNA in situ hybridization on paraffin sections of embryos spanning developmental stages from postimplantation to birth. *Sax2* gene expression can be detected as early as embryonic stage E10.5 in the ventral mesencephalon, metencephalon, and ventral neural tube, as shown in Fig. 1a, panels A to F. The *Sax2* gene continues to be expressed from this embryonic stage onward, predominantly in the ventral neural tube and the mid/hindbrain boundary region, as shown in Fig. 1a, panels G and H and Fig. 1b, panels A to L. In the later stages, E12.5 to E16.5 *Sax2* gene expression in the brain is detected more specifically in the pons and medulla oblongata (Fig. 1b).

**Coexpression analysis of *Sax2* with other developmental and cell-type-specific marker genes.** *Sax2* gene expression occurred in the isthmic organizer (the mid/hindbrain boundary region) (Fig. 1a and b), one of the two major organizers responsible for the development of the brain. This region is also important for the specification of two subsets of neurons, the majority of the dopaminergic neurons and a subset of the serotonergic neurons (21). Dopaminergic neurons are localized in the diencephalon and telencephalon and, most importantly, in the ventral midbrain, in the substantia nigra pars compacta and the ventral tegmental area. The two groups of serotonergic neurons reside in the rostral hindbrain and more caudal regions, respectively. Serotonergic neurons are projected from the raphe nuclei into the central nervous system, with the medium and dorsal raphe nuclei being the two major nuclei projecting into the brain (21). To determine whether *Sax2* expression in the midbrain-hindbrain boundary region colocalizes with dopaminergic or serotonergic neurons, we performed RNA in situ hybridization on adjacent transverse paraffin sections of embryos at stage E13.5. As cell-specific marker genes, we employed serotonin transporter (*ST*) and *Pet1* for serotonergic neurons (4, 37) and *Nurr1*, *Ptx3*, and *tyrosine hydroxylase* (*TH*) for dopaminergic neurons (3, 31, 47). As shown in Fig. 1c, *ST* is expressed in the medial and dorsal raphe nuclei (panels C and D) and *Pet1* is expressed in the medial raphe nucleus (panels E and F), which is in close proximity to *Sax2* expression (panels A and B), suggesting a possible involvement of *Sax2* in serotonin-producing neurons. The marker genes for dopaminergic nuclei are expressed predominantly in the hypothalamus region (panels G to L) and show no overlapping expression pattern with *Sax2*. We also compared the expression patterns of *Sax1* and *Sax2*. As described previously (45) the *Sax1* gene is expressed in the mesencephalon, tegmentum, diencephalon, and pretectum. Comparing adjacent transverse

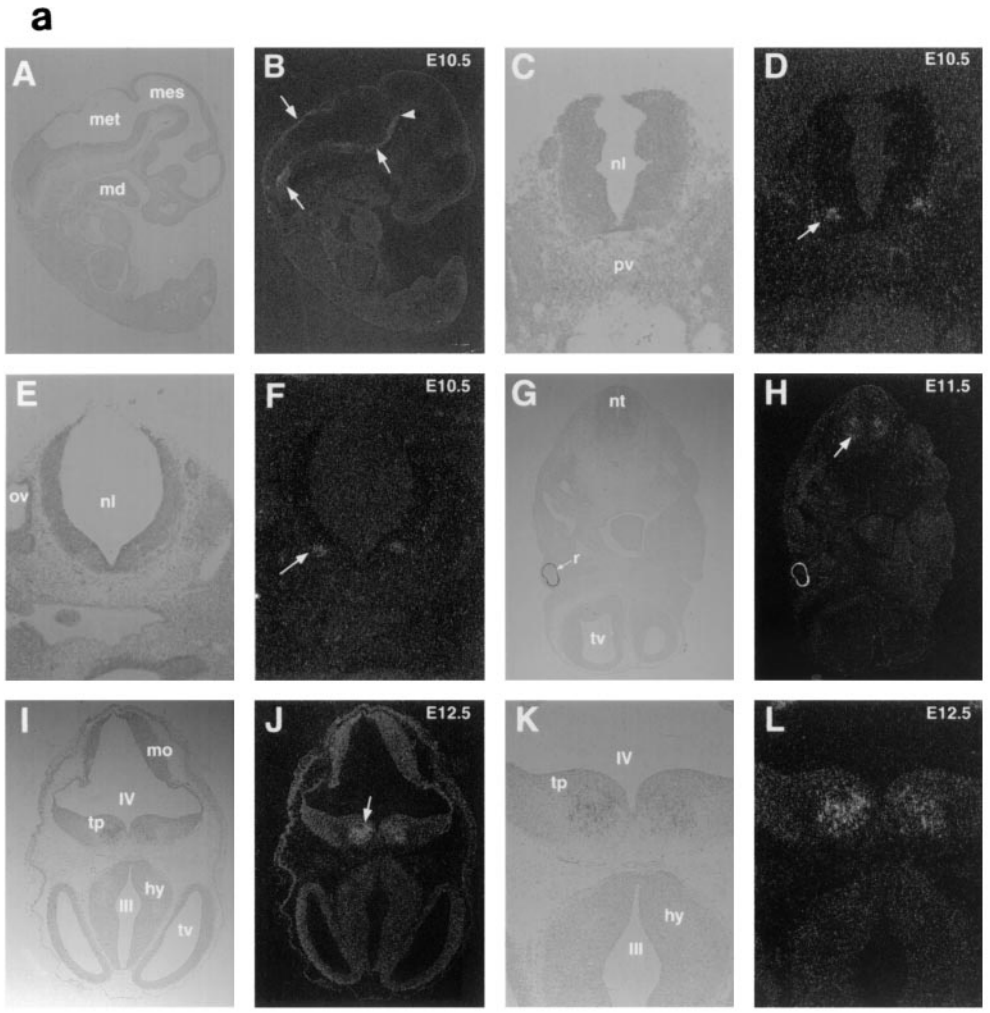


FIG. 1. Analysis of the *Sax2* expression pattern (a and b) *Sax2* expression pattern during embryogenesis. RNA in situ hybridization on sagittal (A and B) and transverse (C to L) paraffin sections of wild-type embryos at embryonic stages 10.5 to 12.5 (a) and stages 14.5 to 16.5 (b) is shown. The embryonic stage is indicated in the upper right corner of each dark-field panel. The first and third columns of panels show light-field microscopy; the second and fourth columns show dark-field microscopy. *Sax2* gene expression already occurs at E10.5 in the ventral mesencephalon, the metencephalon, and the ventral neural tube, as shown in panels A and B on sagittal sections and panels C to F on transverse sections. *Sax2* gene expression remains active at least up to embryonic stage E14.5 in the ventral neural tube (panels G and H) and up to embryonic stage E15.5 in the tegmentum of the pons (b, panels A to D, I, and J). *Sax2* gene expression also occurs in the medulla oblongata, as shown for embryos at E14.5 (b, panels E and F) and E16.5 (b, panels K and L). Panels M to O indicate the angle of sections at the described embryonic stages. Abbreviations: brp, basal regions of pons; ca, cerebral aqueduct; cs, corpus stratum; di, diencephalon; drg, dorsal root ganglion; hy, hypothalamus; l, lens; lv, lateral ventricle; mes, mesencephalon; met, metencephalon; md, mandibular branch of first branchial arch; mo, medulla oblongata; nc, nasal cavity; nl, neural lumen; ns, nasal septum; nt, neural tube; ov, otic vesicle; p, pons; pv, prevertebrae; r, retina; rmo, rostral medulla oblongata; sc, spinal cord; t, telencephalon; th, thalamus; tp, tegmentum of pons; tv, telencephalic vesicle; v, ventral; III, third ventricle; IV, fourth ventricle. (c): Coexpression analysis in the midbrain-hindbrain region. RNA in situ hybridization on adjacent transverse paraffin sections of embryos at stage E13.5 is shown. *ST* and *Pet1* RNA probes are marker genes for serotonergic neurons; *Nurr1*, *Ptx3*, and *TH* RNA probes are marker genes for dopaminergic neurons. The first and third columns show light-field microscopy; the second and fourth column show dark-field microscopy. The *Sax2* gene is expressed in the tegmentum of the pons (panels A and B) in close vicinity to serotonergic neurons as marked by *ST* and *Pet1* (panels C to E) gene expression. *Nurr1*, *Ptx3*, and *TH* are expressed predominantly in the hypothalamus, as shown in panels G to L. Panels M to P show the comparison of the expression patterns of *Sax1* (panels O and P) and *Sax2* (panels M and N). *Sax1* is expressed predominantly in the pons and to a lesser degree in the medulla oblongata (panels O and P). While *Sax1* expression overlaps with the expression of *Sax2* in these areas, *Sax2* exhibits a broader expression pattern. Abbreviations: cc, central canal; drn, dorsal raphe nucleus; hy, hypothalamus; mo, medulla oblongata; mrn, medial raphe nucleus; ST, serotonin transporter mRNA; TH, tyrosine hydroxylase; tp, tegmentum of the pons; tv, telencepahlic vesicle; III, third ventricle; IV, fourth ventricle.

paraffin sections for *Sax1* and *Sax2* gene expression, we found that *Sax1* is expressed strongly in the tegmentum of the pons and to a lesser extent in the medulla oblongata (panels O and P). *Sax2* gene expression also occurs in these areas, but the overall expression pattern for *Sax2* is broader relative to *Sax1* (panels M and N).

**Generating a loss-of-function *Sax2* null allele by *lacZ* knock-in.** The loss-of-function mutant for the *Drosophila S59/slouch*

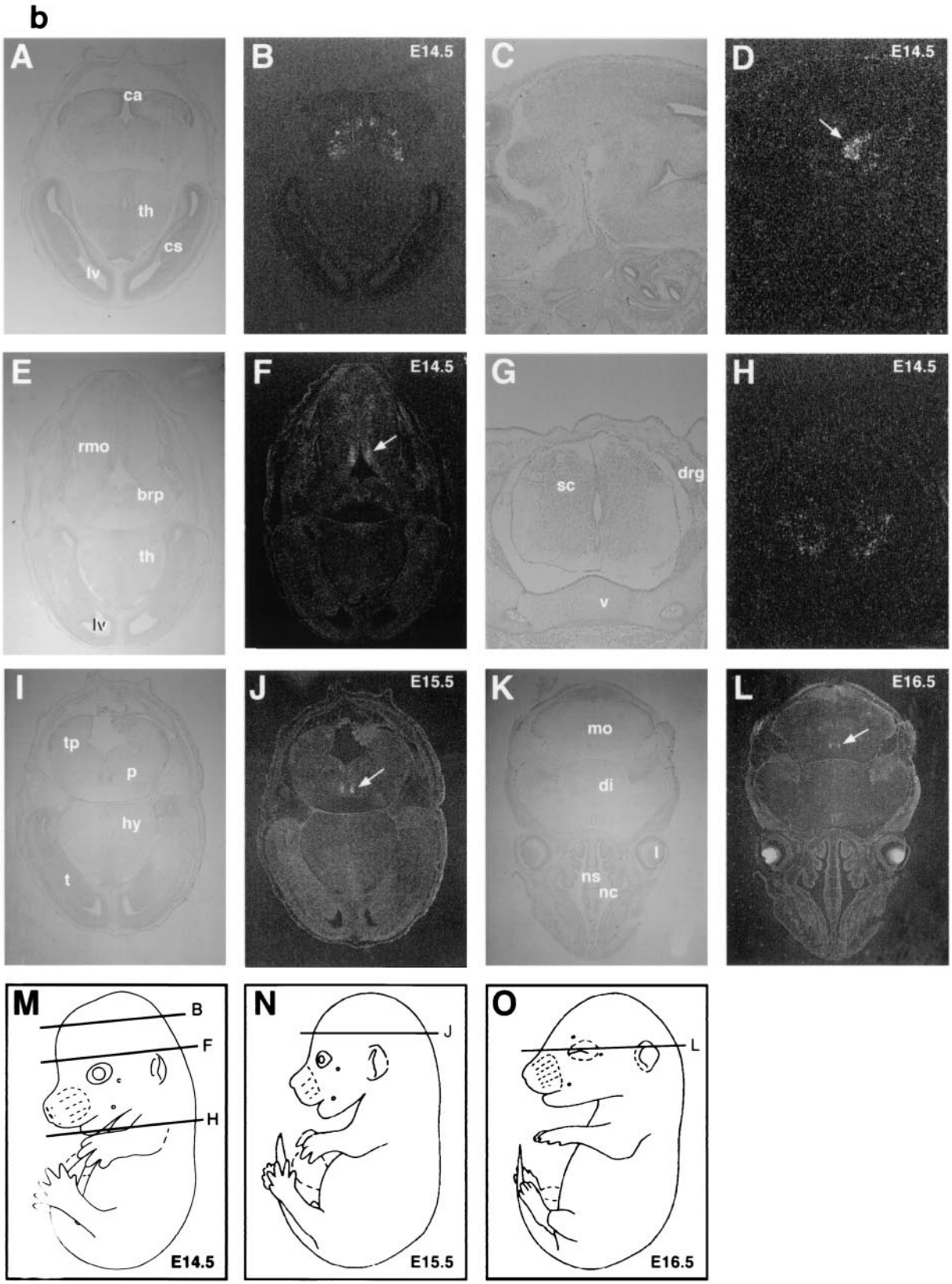


FIG. 1—*Continued*

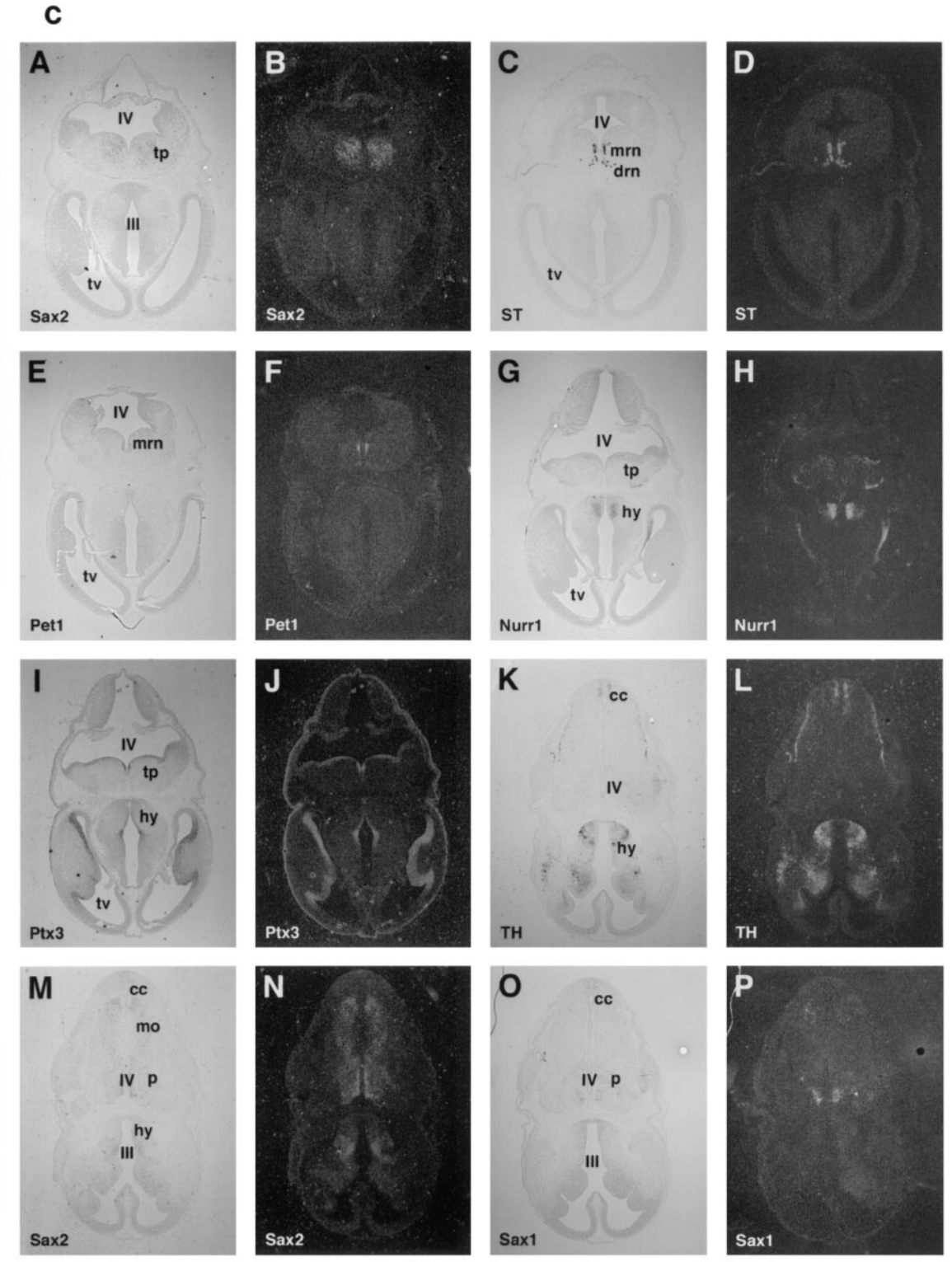


FIG. 1—*Continued*

gene causes 90% death at different stages during embryogenesis (25). In contrast to the *Drosophila* mutant, the *Sax1* loss-of-function null mice do not exhibit an overt phenotype (P. Gruss, personal communication). This stark discrepancy between the fly and mouse phenotypes might be explained by the overlapping expression patterns of murine *Sax1* and *Sax2* and a possible functional equivalence between these genes. To determine the role of *Sax2* during development, we generated

a loss-of-function mutant mouse line. Cloning and the chromosome localization of *Sax2* were previously described (7). An 18-kb *Not*I fragment containing the *Sax2* gene was cloned into the *Sal*I site of the pTZ18U vector, resulting in construct p1083. Comparing a sequence of more than 6 kb obtained from construct p1083 with existing genomic databases, combined with our cDNA sequence analysis, predicted an mRNA of 1,074 bp derived from four exons containing a homeobox located in the third exon and an N-terminal conserved element, the EH1 motif, involved in transcriptional repression located in the second exon (44). The predicted mRNA was confirmed by a 744-bp cDNA clone obtained by RT-PCR using mRNA from embryos at stage E11.5 and specific primers corresponding to sequences in the second exon spanning 70 bp upstream of the cloning site for the targeting vector and downstream of the homeobox in the third exon (data not shown).

To generate the *Sax2* targeting vector, we inserted an *IRESlacZ/floxedneo* cassette into the *Bsp*EI sites located in the second exon and second intron of the *Sax2* gene (Fig. 2A). The targeting vector was electroporated into ES (R1) cells, and 27 of 192 ES clones were confirmed positive by Southern blotting. Four of these ES clones were microinjected into blastocysts to generate chimeras. Of 24 chimeric males, 16 transmitted the gene through the germ line.

**Phenotype of *Sax2* null mice.** *Sax2* heterozygous pups were indistinguishable from wild-type animals at all stages of development and postnatal life. Intermating of *Sax2* heterozygous animals yielded homozygous mutant newborns in the predicted Mendelian ratios. At birth, homozygous pups were indistinguishable from their wild-type or heterozygous littermates up to 2 days postpartum; subsequently, homozygous pups were easily recognizable owing to their smaller size (Fig. 2C and D). The homozygous pups continued to grow much more slowly than their littermates, and 85% of the homozygous pups died within the first 3 weeks of birth (Fig. 2C and data not shown). Although homozygous mice were runted, they did not exhibit any obvious abnormal behavior or motor skills. All *Sax2* null pups showed normal suckling behavior, and milk was found in their stomachs. At 2 or 3 days prior to their death, *Sax2* null pups became lethargic and showed signs of wasting. The few homozygous animals surviving to adulthood were fertile, but all the offspring from homozygous intermatings died within 4 days postnatally.

We examined whether the mutant mice exhibited gross behavioral or neurological differences by performing several tests including the contact righting test and reaching reflex test for neurological reflexes, the jar-circling test for hyperactivity and adaptation to a new environment, the gait test for motor abilities, and the forelimb grip test for neuromuscular abnormalities. In all cases, with the exception of the forelimb grip test, the mutants did not show any statistically significant differences in their behavior or performance. The grip test revealed that the *Sax2* null mutants were able to grip the suspension bar but could not hold on as long as the wild-type pups. While wild-type mice could hang from the bar for up to 90 s, the maximum time for the mutants never exceeded 30 s. From our observations, it is unclear at present whether this difference in behavior is due to neuromuscular abnormalities or is related to a secondary effect owing to an evident malnutrition of the *Sax2* null animals.

To exclude the notion that the phenotype we obtained was influenced by the presence of the *PKGneo* selection cassette, we intermated *Sax2* heterozygous animals with a Cre recombinase-expressing mouse line (34) that exerts Cre activity as early as the zygote-blastomere stage of mouse embryogenesis. Loss of the *neo* gene in the resulting heterozygote offspring was confirmed by Southern blotting. *Sax2* heterozygotes segregating free of the Cre transgene were selected in subsequent generations. Intermatings of *Sax2* heterozygous animals carrying only the *IRESlacZ* reporter (Fig. 2A) produced offspring exhibiting the same phenotype as described above (data not shown), indicating that the *PGKneo* cassette did not contribute to the observed phenotype.

**Analysis of *lacZ* gene expression in heterozygous and homozygous *Sax2* embryos.** To further examine the expression pattern of *Sax2* and to analyze possible effects of the loss-of-function mutation, we compared the *lacZ* expression pattern of heterozygous and homozygous embryos by β-galactosidase staining. Embryos were collected at stages E8.5 to 14.5 for β-galactosidase staining as previously described (14). Similar to the RNA in situ hybridization results, β-galactosidase staining revealed expression of the *Sax2* gene in the ventral neural tube, the hindbrain, the midbrain, the eyes, and the apical ectodermal ridge (AER) (Fig. 3). *lacZ* gene expression was first detected at stage E10 in the AER of the forelimb (data not shown). At embryonic stage E10.5, β-galactosidase staining was also detected in the AER of the hindlimb (Fig. 3B and D) and to a lesser degree in the ventral neural tube (Fig. 3B). The same expression pattern was observed in embryos at stage E11.5 (Fig. 3E to H). At embryonic stage E13.5, *lacZ* gene expression still persisted in the AER remnant, but at that time it was found only in the cells distal to the region of the developing digits and not in the interdigital region (Fig. 3I and J, inserts). *Sax2*-directed *lacZ* expression in the neural tube occurred in two distinct columns located on either side of the midline of the neural tube (Fig. 3K and L). Expression of *lacZ* in the lens and hindbrain was first seen at embryonic stage E12.5 (data not shown) and showed its highest expression level at stage E13.5 (Fig. 3K to P). *lacZ* gene expression in the limb and neural tube was stronger in homozygous than in heterozygous embryos, which would be expected owing to gene dosage effects; expression in the midbrain-hindbrain boundary region and especially in the eye was strikingly down regulated in the *Sax2* homozygous nulls (Fig. 3K, L, O, and P). In addition to *lacZ* expression in the ventral neural tube, we detected β-galactosidase staining in the ventral midbrain of *Sax2* homozygous null embryos but not in the heterozygous embryos (Fig. 3I and J). Unlike in the eye and midbrain-hindbrain boundary region, where *lacZ* gene expression was down regulated in the *Sax2* null embryos, in the ventral midbrain *lacZ* expression was up regulated in the *Sax2* null homozygous embryo. β-Galactosidase staining was still detected at embryonic stage E14.5 and later in all the tissues described above (data not shown) in a manner similar to the RNA in situ hybridization results for *Sax2*.

**Determination of *Sax2* gene function.** The *Sax2* gene expression pattern and the postnatal lethal phenotype of the loss-of-function mutant suggested a critical role for the *Sax2* gene during embryogenesis and postnatal maturation. *Sax2* loss-of-function mutants exhibit a highly reduced growth rate starting

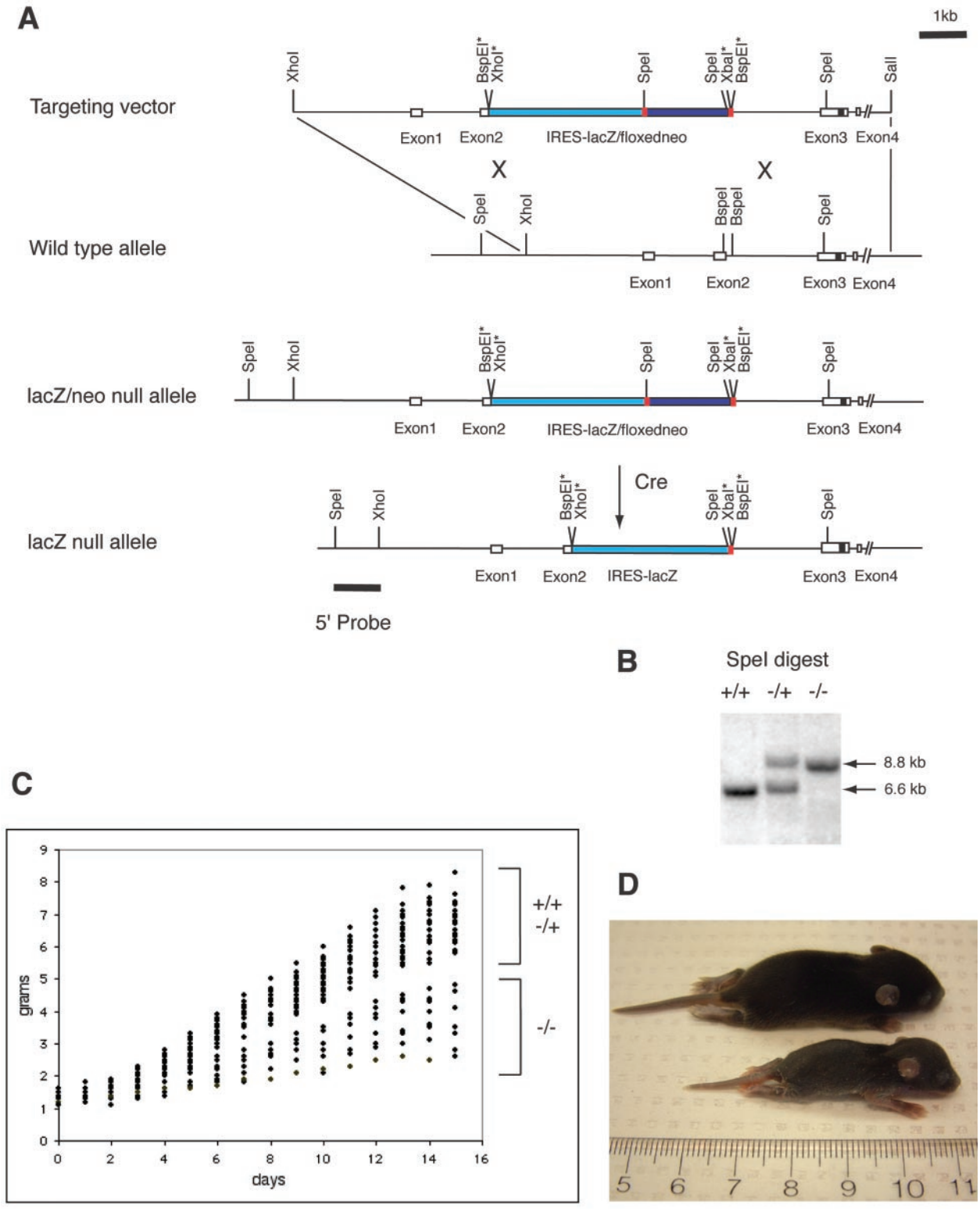


FIG. 2. Generation of a loss-of-function mutant with an *Sax2* mutation and overview of the phenotype. (A) Targeting vector, wild-type, *lacZ*/*floxedneo* null, and *lacZ* null alleles. The targeting construct has the *IRESlacZ*/*floxedneo* cassette inserted into the *Bsp*EI sites located in the second exon and intron of the *Sax2* gene. This mutant allele produces β-galactosidase and a nonfunctional truncated *Sax2* protein. Intermating of heterozygous *Sax2* transgenic mice with wild-type mice expressing Cre recombinase resulted in the deletion of the *neo* gene. Asterisks indicate modification of restriction sites during the cloning process, which generate a polymorphism in the mutant allele. The solid black box indicates the homeobox, the light blue box indicates the IRESlacZ construct, the dark blue box indicates the *neo* gene, and red boxes indicate *lox* sequences. (B) Southern blot analysis of genomic DNAs from *Sax2* wild-type, *Sax2* heterozygous, and *Sax2* homozygous offspring. Wild-type *Sax2* DNA produces a band of 6.6 kb, while *Sax2* homozygous DNA produces a band of 8.8-kb using an external 5′ probe as indicated in panel A. (C) Growth curve comparing *Sax2* wild-type, *Sax2* heterozygous, and *Sax2* homozygous offspring. Data represent the growth curves for 34 pups derived from four litters. Wild type, $n = 13$; Sax2 heterozygous, $n = 14$; Sax2 homozygous, $n = 7$. (D) Comparison of *Sax2* wild-type (top) and *Sax2* homozygous (bottom) pups at day 10 postnatal.

at birth and ending in the death of 85% of the homozygous offspring within the first 3 weeks. We also observed that endogenous *Sax2* or *lacZ* from the mutant allele is regulated differently in different tissues, e.g., down regulated in the eyes and upregulated in the ventral midbrain (Fig. 3). Examining the developing limb in the *Sax2* null embryos, we found no obvious difference compared to wild-type embryos. Staining of dissected forelimbs and hindlimbs derived from wild-type and homozygous 8-day-old pups with Alizarin Red and Alcian Blue (52) confirmed delayed but normal development of the limb skeleton in the *Sax2* mutants (data not shown). The expression pattern of the eye, the neural tube, and the brain was further analyzed by RNA in situ hybridization employing specific marker genes for each tissue.

**Analysis of *Sax2* gene expression in the developing eye.** *Sax2* gene expression in the developing eye is first detected at embryonic stage E12.5 (data not shown), coinciding with the onset of primary lens fiber cell differentiation (13), and *Sax2* expression becomes highest at embryonic stage E13.5, as determined by β-galactosidase staining (Fig. 3M to P). Furthermore, transverse sections of paraffin-embedded β-galactosidase stained embryos at stage E13.5 revealed that *lacZ* expression occurs in the proximal region of the lens fibers (Fig. 4A and B), with a high expression level in heterozygous embryos and a very weak expression level in homozygous embryos. Performing RNA in situ hybridization using a probe corresponding to the 5′ end of the *Sax2* mRNA (which is present in both the wild-type and *Sax2* null alleles), we confirmed the expression pattern and the down regulation of *Sax2* gene expression in the developing *Sax2* null eye (Fig. 4C and D), suggesting a positive autoregulation mechanism for *Sax2* gene expression in these cells. To further determine whether other genes involved in the development of the eye, in particular in lens development, are affected by the *Sax2* loss-of-function mutation, we analyzed several genes involved in the development of the eye, specifically in the development of the lens fibers by RNA in situ hybridization, as shown in Fig. 4E to P. While *Alk6* (13), *Bmp4* (15), and *Fgf1* (9) were expressed predominantly in the lens fiber cells (Fig. 4E to J), *Hmx1* (53), *Ncadherin* (55), and *Pax6* (8) were also expressed in the neural retina (Fig. 4K to P). All the genes used for the RNA in situ hybridization show an overlapping expression pattern with the *Sax2* gene in the lens fiber cells, but, surprisingly, none exhibits any change in expression in the *Sax2* null embryos, suggesting that *Sax2* lies either downstream or in a separate developmental pathway from the genes investigated here.

**Analysis of *Sax2* gene expression in the ventral neural tube.** RNA in situ hybridization experiments on wild-type embryos and β-galactosidase staining revealed *Sax2* gene expression in the ventral neural tube as early as E10.5; the highest level of expression was achieved by E13.5. Transverse paraffin sections of β-galactosidase-stained embryos at E13.5 revealed four columns in the ventral neural tube. The specification of different neuronal cell types in the ventral neural tube results from a range of signals and interactions among different homeobox genes. These activities are mediated in part by the secreted protein *Shh*, which is produced by the notochord and the floor plate (reviewed in references 5, 27, and 38). At the time of cellular differentiation in the ventral neural tube, the combinatorial expression of homeodomain proteins identifies five groups of progenitor cell types; V0, V1, V2, MN, and V3. These homeodomain proteins can be divided into two classes; members of class I are repressed at a certain concentration of *Shh*, and class II homeodomain proteins are dependent on *Shh* expression for activation (reviewed in references 5 and 24). The data we obtained from the *lacZ* expression analysis suggested that *Sax2* gene expression occurs in the vicinity of the motor neurons and the V2 interneurons. The question arose whether *Sax2* might itself be a member of one of these classes and/or might interact with other members and therefore be involved in the neuronal differentiation process in the ventral neural tube. To answer this question and to further determine the specific expression domain of *Sax2*, we performed RNA in situ hybridization experiments with genes expressed in the different neuron cell types, namely, *Engrailed1* (*En1*) (30) in the V1 group, *HB9* (2) and *Islet 2* in the MN group (22), *Lim3* (50) in both the MN and V2 groups, and *Pax6* (12) in the V0, V1, V2, and MN groups. *HB9* and *Islet2* genes were expressed laterally and ventrally compared to the *Sax2* gene expression pattern, excluding the MN group as expression domain for *Sax2* (Fig. 5C, D, and G to J). *Lim3* was also expressed in these areas, but in addition its expression occurred more dorsally and overlaps with *Sax2* gene expression (Fig. 5C, D, K, and L). While *Pax6* gene expression also showed some overlap with *Sax2* gene expression (Fig. 5M and N), neither *En1* nor *Shh* gene expression colocalized with *Sax2* gene expression (Fig. 5E, F, O, and P). These results suggest that *Sax2* gene expression is restricted to the V2 interneuron subgroup. Surprisingly, none of the genes examined here showed a difference in their expression pattern in the *Sax2* null mutant relative to wild-type embryos, suggesting that there is no direct regulatory interaction between these genes and *Sax2*. However these data do not exclude a function for *Sax2* in the differentiation of the ventral neural tube downstream of these genes and, more specifically, in the further differentiation of the V2 interneurons into its V2a and V2b subgroups, possibly by interacting with homeobox gene Chx10 and/or the zinc finger transcription factors GATA2/3 (reviewed in reference 38).

**Analysis of *Sax2* gene expression in the brain.** In addition to *Sax2* gene expression in the limbs, eyes, and ventral neural tube, *Sax2* is expressed in the midbrain-hindbrain boundary region and in two very distinct areas in the ventral midbrain. Coronal paraffin sections of β-galactosidase-stained brains of embryos at stage E18.5 revealed *lacZ* gene expression ventral to the fourth ventricle. While β-galactosidase staining was very strong in the homozygous brain, there was, interestingly, no corresponding staining in the same area in the heterozygous brain (Fig. 6A and B). To determine whether this observation resulted from the differential accumulation of the β-galactosidase protein or whether *lacZ* gene expression is up regulated in the *Sax2* null mutants, we performed RNA in situ hybridization on coronal section of wild-type, heterozygous, and homozygous embryonic brain sections of stage E18.5 embryos, using a probe corresponding to the 5′ end of *Sax2* mRNA which is present in both the *Sax2* wild-type and mutant alleles. While no signal was detected on sections of the wild-type brain (Fig. 6C) and next to no signal was detected in the heterozygous brain (Fig. 6E), the homozygous brain exhibited very strong *Sax2* expression (Fig. 6F), suggesting either negative autoregulation of *Sax2* gene expression in this tissue or possibly the migration

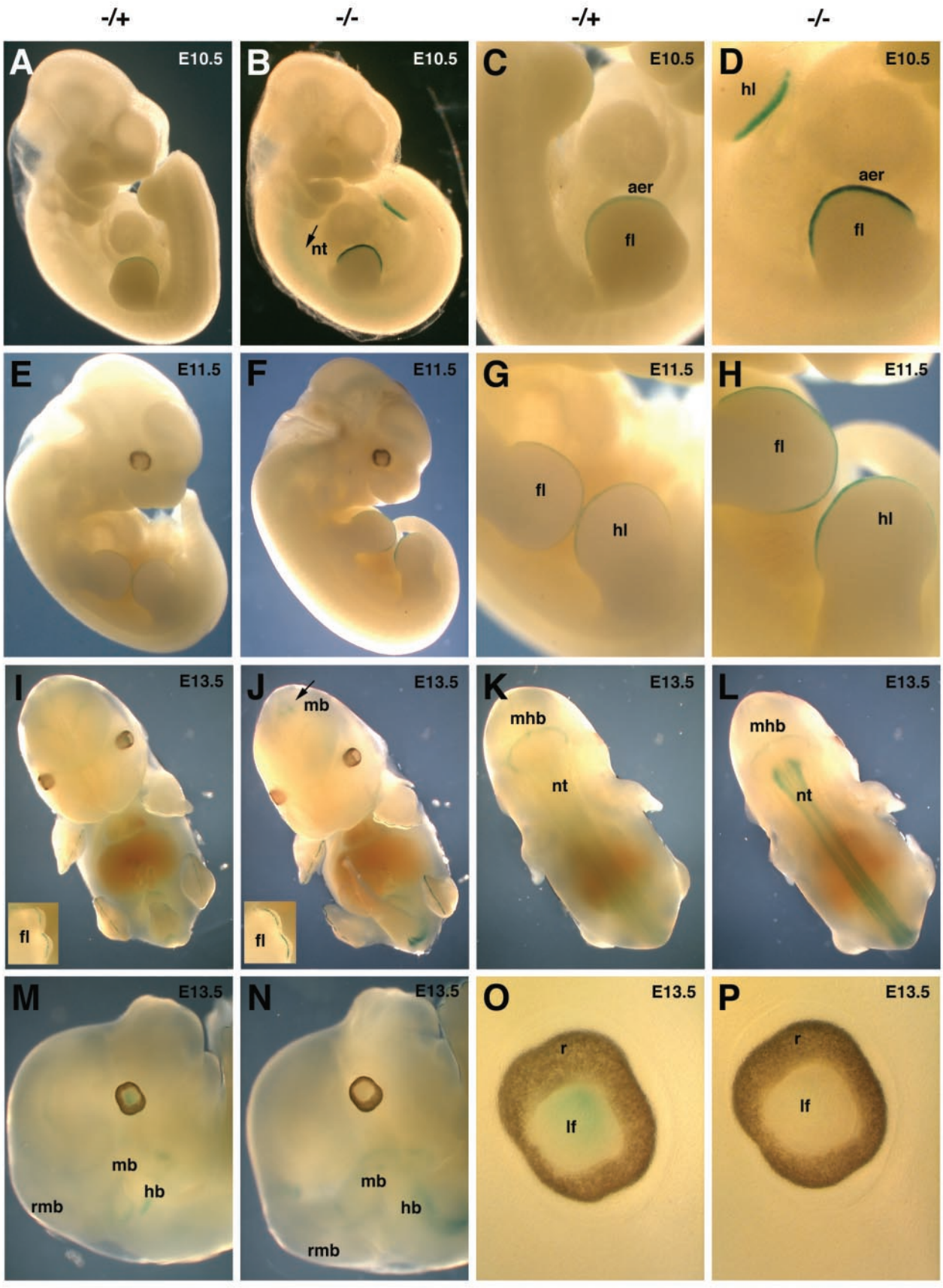
-/+
-/-
-/+
-/-
A
E10.5
B
E10.5
nt
C
E10.5
aer
fl
D
E10.5
hl
aer
fl
E
E11.5
F
E11.5
G
E11.5
fl
hl
H
E11.5
fl
hl
I
E13.5
fl
J
E13.5
mb
fl
K
E13.5
mhb
nt
L
E13.5
mhb
nt
M
E13.5
mb
hb
rmb
N
E13.5
mb
hb
rmb
O
E13.5
r
lf
P
E13.5
r
lf

FIG. 3

of *Sax2* gene-expressing cells into this region in the mutants. Our data suggest that it is more likely that *Sax2* gene expression is undergoing autoregulation (release from repression) in this tissue and that the extent of repression is dependent on the allele-mediated dosage of Sax2 protein present in individual cells, since intermediate levels of *Sax2* mRNA expression were observed in the heterozygotes relative to the wild-type and *Sax2* null embryos.

Both the *lacZ* gene expression pattern and RNA in situ hybridization data suggest the colocalization of *Sax2* gene expression with two midbrain nuclei, the red nucleus and the oculomotor nucleus. Both nuclei derive from the medial arc, and both are part of the motor system, but they have very different functions (1). The oculomotor nucleus contains motor neurons that control eye movement and the parasympathetic regulation of accommodation and pupil contraction. In contrast, the red nucleus does not contain motor neurons but is a cerebellum-related nucleus mediating motor cortex and cerebellar outflow to the spinal cord in the control of limb movements. To determine whether *Sax2* expression occurs in either of these nuclei, we performed RNA in situ hybridization experiments with *Emx2* as a marker gene for the red nucleus (1, 6) and *Motopsin* as a marker gene for the oculomotor nucleus (1, 23). Neither of these two genes showed an overlapping expression pattern with *Sax2*; in fact, *Motopsin* was expressed in the cells immediately lateral and adjacent to the *Sax2*-expressing cells along the midline (Fig. 6G, H, I, J, and M to P). Furthermore, our data show that there is no significant difference in the expression pattern of *Emx2* and *Motopsin* in the *Sax2* null embryos relative to the wild-type ones. Comparing the *Sax2* gene expression pattern to other cell groups described in the midbrain, we also examined marker genes for the dorsal and lateral thalamus. We employed *SorCS2* (42) and *Pax6* (39) as markers for cells of the dorsal thalamus and *Calretinin* (17) as markers for cells for the lateral dorsal thalamus. While *SorCS2* and *Calretinin* were not expressed in the same cells where *Sax2* expression occurred, *Pax6* exhibited overlapping expression with *Sax2* (Fig. 6K, L, and Q to T). The results we obtained from the *SorCS2* and *Calretinin* expression analysis rule out the dorsal and lateral dorsal thalamus as expression sites for *Sax2*. This does not contradict our results from the *Pax6* gene expression analysis, since *Pax6* has a broader expression pattern than *SorCS2* and *Calretinin* and is expressed in many additional cell groups in the developing brain. Even so, *Pax6* and *Sax2* gene expression do overlap in the ventral midbrain; and within these cells there is no change in expression of *Pax6* in the *Sax2* null brain, indicating that *Pax6*, although involved in many developmental processes, is not a direct downstream target of *Sax2* function.

As we have shown in Fig. 1c, panels A to F, *Sax2* is expressed in close proximity to the *ST* and *Pet1* genes, two marker genes for serotonergic neurons. Among the functions ascribed to serotonergic neurons are the regulation of anxiety, affection, aggression, drug abuse, and food uptake (reviewed in reference 16). Because of the close proximity of *Sax2* gene expression to serotonergic neurons, it is possible that the loss of the *Sax2* gene product might affect food uptake and therefore contribute to the observed phenotype. We performed an RNA in situ hybridization analysis on transverse paraffin sections of embryos at stage E16.5 to further examine the cells expressing *Sax2* and to explore the possibility of an interaction between *Sax2* and these serotonergic genes. *Sax2* gene expression occurred in the midbrain-hindbrain boundary adjacent to cells expressing *ST* (Fig. 6U to X). While *Sax2* gene expression was down regulated in the homozygous null brain compared to the wild type (which concurs with the *lacZ* expression data shown in Fig. 3K and L), there was no significant difference in the level of expression of the *ST* gene in the *Sax2* nulls. These data suggest that *Sax2* is not directly interacting with *ST*; however, they do not exclude the possibility that there is an interaction between *Sax2*-expressing cells and serotonergic neurons, since it is possible that the decrease in the level of *Sax2* protein in the homozygous mutant in the midbrain-hindbrain boundary region acts downstream from *ST* or possibly has an effect on other factors involved in the sensory neuron pathway.

## DISCUSSION

***Sax2* null allele phenotype.** Many homeobox genes play an important role during embryogenesis, especially in the development of the nervous system. We have shown that the homeobox-containing gene *Sax2* is expressed early during development of the limbs, the eyes, the ventral neural tube, the ventral midbrain, and the midbrain-hindbrain boundary region. This expression pattern implied an important function for *Sax2* during embryogenesis; however, *Sax2* null pups were born in a Mendelian distribution, suggesting that the loss of the *Sax2* gene product does not critically affect embryonic viability. Shortly after birth, *Sax2* null pups exhibited a reduced growth rate, resulting in pups half the size of their littermates and premature death of 85% of the pups within the first 3 weeks following birth. The reduced growth rate might result from behavioral or physiological defects, yet *Sax2* null pups showed no difference in their suckling behavior and in overall mobility compared to their littermates, most probably ruling out the notion that severe behavioral defects are responsible for the phenotype. Newborn *Sax2* null allele mutants are indistinguishable from their littermates and develop their distinct

FIG. 3. *lacZ* gene expression analysis in *Sax2* heterozygotes and homozygotes during embryogenesis. *Sax2* heterozygous and homozygous embryos at stages E10.5, E11.5, and E13.5 were stained for *lacZ* gene expression as whole mounts. (A to H) At embryonic stage 10.5 and E11.5, expression is strongest in the AER and to a lesser degree in the ventral neural tube. (I and J) At E13.5, *lacZ* gene expression still persists in the AER remnant but is restricted to the cells distal to the digits and is not found in the distal interdigital zone. (K and L) *lacZ* gene expression is also found in the neural tube and the midbrain-hindbrain region. In the limb and neural tube, no difference in the intensity of β-galactosidase staining is observed. (M to P) The *lacZ* gene is also expressed in the eyes, the lens fiber, of *Sax2* heterozygote embryos (M and O) but not in *Sax2* homozygous embryos (N and P). (J) In addition to the midbrain-hindbrain region, β-galactosidase staining is found in the ventral midbrain (J). Abbreviations: aer, apical ectodermal ridge; fl, forelimb; hb, hindbrain; hl, hindlimb; lf, lens fiber; mb, midbrain; mhb, midbrain-hindbrain boundary; nt, neural tube; rmb, roof of midbrain.

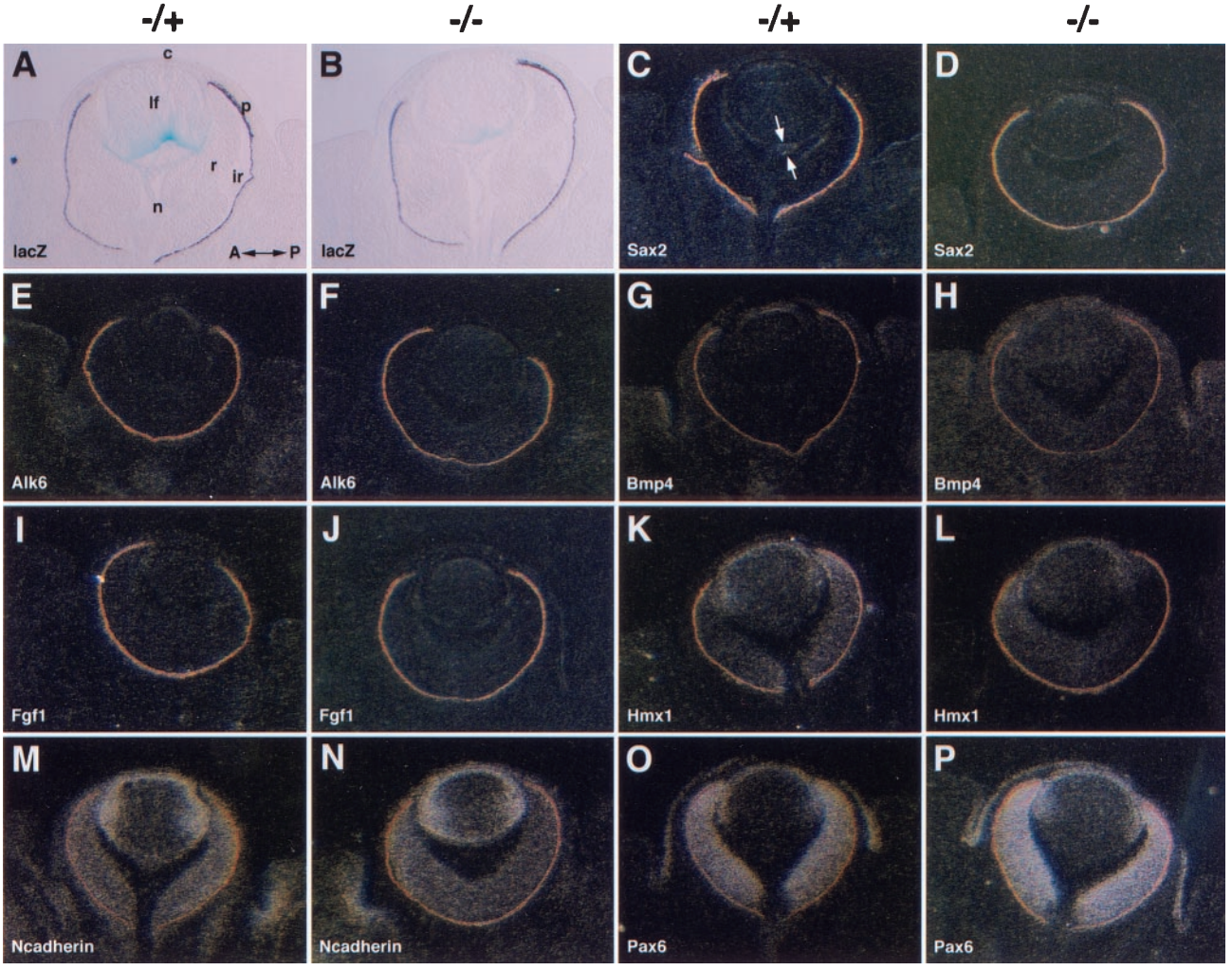


FIG. 4. Analysis of *Sax2* expression in the developing eye. (A and B) Sections of β-galactosidase whole-mount staining of embryo stage E13.5 followed by paraffin embedding. (C to P) RNA in situ hybridization on transverse paraffin sections of embryo stage E13.5. RNA in situ hybridization using a probe corresponding to the 5′ end of *Sax2* RNA shows high levels of expression in the heterozygous embryo (C) and very little expression in the homozygous embryo (D), confirming the data obtained by β-galactosidase staining (A and B). The expression limits of *Sax2* are indicated by arrows in panel C, and are reduced in panel D. Panels E to P show the in situ hybridization results for genes involved in the development of the eye, especially in the development of the lens fiber. None of the genes examined showed any difference in their expression pattern between heterozygous and homozygous embryos. Abbreviations: A, anterior; c, cornea; ir, intra-retinal space; lf, lens fiber; n, nerve; p, pigment; P, posterior; r, retina.

wasting phenotype only postnatally, suggesting possible changes in their metabolism and/or environment after birth.

Mutants with mutations of the *growth hormone receptor/binding protein* gene (56), *Pax7* gene (46), *VGF* gene (18), and *Twist* genes (49) exhibited a similar phenotype of reduced postnatal growth rate. Mice lacking the *growth hormone receptor/binding protein* gene were born indistinguishable from their littermates, but by 3 weeks after birth the homozygous mice were significantly smaller. Aside from their regressing growth rate, these mice were viable and fertile. Growth retardation in this case might result from the increased levels of growth hormones in serum in the mutant mice (56). Mice lacking the basic loop-helix transcription factor *Twist* are similar to the *Sax2* mutants, exhibiting postnatal growth retardation and dying within 3 to 4 weeks after birth. The phenotype appears to be caused by the loss of a negative-feedback mechanism associating *Twist* protein with the NF-κB subunit *p65* and the repression of the cytokine promoters (49). At birth, cytokine expression, especially the expression of the cytokines tumor necrosis factor alpha and interleukin-1β is very high, possibly reflecting an immune response to the stress of delivery and changes in the environment after birth (40). The *Twist* gene product down regulates this high expression rate of cytokines, and cytokine levels remain unnaturally high in *Twist* null mutants, leading to premature death (49). It is possible that *Sax2* gene expression is required in a similar way to *Twist* gene expression in order to adjust the expression of target genes to the changed metabolism and/or environment after birth.

***Sax2* gene expression and different mechanisms of autoregulation depending on cell type.** Correct spatiotemporally regulated expression of transcription factors is crucial during development. Misexpression of developmental control genes can

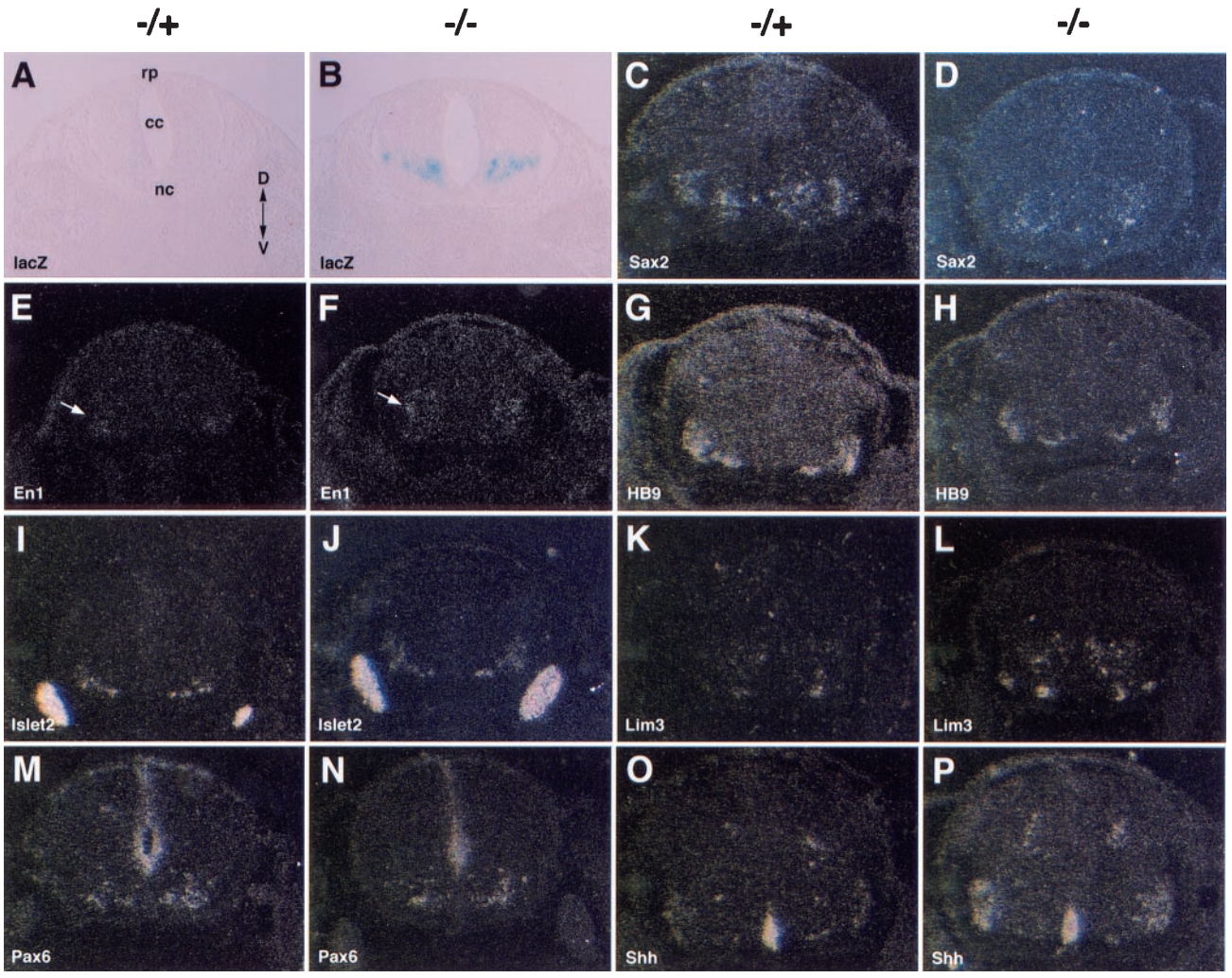


FIG. 5. Analysis of *Sax2* expression in the ventral neural tube. (A and B) Sections of β-galactosidase whole-mount staining of embryo stage E13.5 followed by paraffin embedding. (C to P) RNA in situ hybridization on transverse paraffin sections of embryo stage E13.5. The β-galactosidase staining results suggest a strong increase in *lacZ* gene expression of in the ventral neural tube of the homozygous embryo (A and B). RNA in situ hybridization using a probe corresponding to the 5′ end of the *Sax2* RNA reveals equal expression levels of these cells in both types of embryos, indicating that β-galactosidase levels may not always reflect corresponding mRNA levels (C and D). Panels E to P show in situ hybridization experiments using marker genes for motorneurons, *HB9*, *Islet2*, and *Lim3* (G to L), ventral interneurons, *En1* (E and F), indicated by arrows, and genes more generally involved in the patterning of the ventral neural tube such as *Shh* and *Pax6* (M to P). *Lim3* (K and L) and *Pax6* (M and N) expression patterns overlap with the *Sax2* (C and D) expression domain. None of the genes examined here significantly changed their expression pattern between heterozygous and homozygous embryos. Abbreviations: cc, central canal; D, dorsal; nc, notochord; rp, roof plate; V, ventral.

lead to severe defects and premature death. Analysis of *Sax2* gene expression revealed different regulatory mechanisms in different tissues during embryogenesis, suggesting an important function for *Sax2* during development. While the level of *Sax2* gene expression does not change in the limbs and the neural tube of *Sax2* mutants, *Sax2* expression is down regulated in the *Sax2* null midbrain-hindbrain boundary region and most obviously in the *Sax2* null eyes. In contrast, no *Sax2* gene expression is observed in the ventral midbrain in wild-type mice, but a very high *Sax2* expression level is observed at both the *Sax2* RNA and the β-galactosidase levels in the null mutant, confined to two distinct areas of the ventral midbrain. Both these expression patterns suggest autoregulation of the *Sax2* gene by a negative-feedback (repression) mechanism in the ventral midbrain and a positive-feedback (activation) mechanism in the midbrain-hindbrain boundary region and the eyes. The specific regulation of *Sax2* gene expression in multiple tissues implies diverse roles for the *Sax2* gene during development.

Autoregulation in either a positive- or negative-feedback manner has been shown for several homeobox genes in *Drosophila* and mammals. The organizer-specific homeobox gene *goosecoid* is one example of autoregulation by a negative-feedback mechanism. *goosecoid* contains two palindromic goosecoid binding elements, DE and PE, in its promoter region. It was shown in *Xenopus* embryos and mouse P19 teratocarcinoma cells that *goosecoid* protein binds the more proximal element and represses its own transcription. It is suggested that

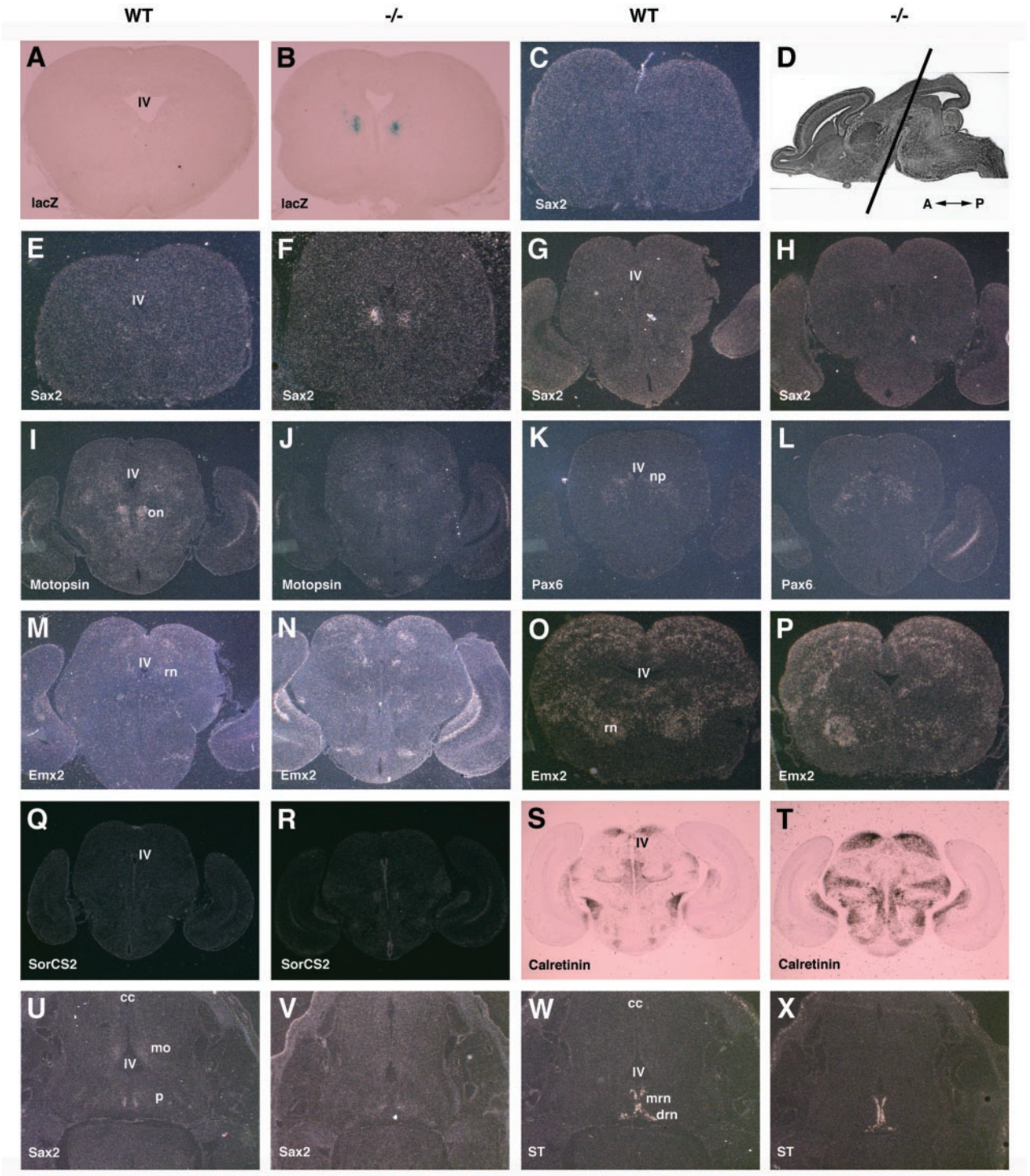


FIG. 6. Analysis of *Sax2* expression in the embryonic brain. (A and B) Sections of β-galactosidase staining of embryonic brains at stage E18.5. (C and E to T) RNA in situ hybridization on coronal paraffin sections of embryonic brains at stage E18.5. (U to X) RNA in situ hybridization on transverse paraffin sections of embryo stage E16.5. (D) Schematic sagittal view of an embryonic brain at stage E18.5. In this figure, WT indicates both +/+ and +/− embryos. β-Galactosidase staining of embryonic brains revealed high expression of the *lacZ* gene in the midbrain ventral of the fourth ventricle of the homozygous embryo (B); no expression was detected in this area in the heterozygous brain (A). These results were confirmed by RNA in situ hybridization using a probe corresponding to the 5′ end of *Sax2* RNA; no hybridization signal was detected in the wild-type brain (C), and a very weak signal was detected in the heterozygous brain (E), while the homozygous brain exhibited a very strong signal (F). To determine the specific region of *Sax2* expression, marker genes for the oculomotor nucleus (I and J), red nucleus (M to P), and thalamus (Q to T) were employed as well as *Pax6* (K and L). Panels U to X show a comparison of *Sax2* and *ST* expression patterns in heterozygous and homozygous brains. Abbreviations: A, anterior; cc, central canal; drn, dorsal raphe nucleus; mo, medulla oblongata; mrn, medial raphe nucleus; np, nucleus proprius of posterior commissure; on, oculomotor nucleus; p, pons; P, posterior; rn, red nucleus; ST, serotonin transporter mRNA; IV, fourth ventricle; WT, +/+ and +/− embryos.

specific cofactors are required to inhibit repressor activity and lead to increased transcription (10). Several members of the *Hox* gene family are regulated in a positive-feedback mechanism. *Hox* genes are very important in the patterning of the anteroposterior axis and in the development of the nervous system. Different *Hox* genes are expressed in a specific spatiotemporal order to establish the segmentation of the hindbrain, and it has been shown, especially for *Hoxa3* and *Hoxa4*, that cross- and auto-regulation of these genes is required for their function (29, 36). Further studies are required to determine the *cis*-acting DNA elements involved in the positive- and negative-feedback mechanisms for the *Sax2* gene.

**Repressive role for *Sax2* during development of the central nervous system.** Homeobox genes are critical transcription factors involved in the regulation of many processes during development by either activation or repression of target genes (reviewed in reference 28). The specification of their regulatory function depends on interactions with specific cofactors. As we have shown above, *Sax2* is regulating its own expression in a positive- and negative-feedback mechanism depending on the specific cell type examined. How does *Sax2* gene regulation relate to its function, and how does it modulate the expression of other genes? The *Sax2* gene, as well as the *Sax1* gene, contains, in addition to the homeobox, a repressor element, the EH1 motif (44), upstream of the homeobox. This motif is also present in all members of the *Engrailed*, *Msh*/*msx*, *NK2*, and *goosecoid* homeoprotein classes (48). It was shown that the EH1 motif plays an important role in the patterning of the ventral neural tube. The ventral neural tube is divided into five neural subgroups that are determined by two classes of homeobox genes; class I is repressed by specific *Shh* protein concentrations, while class II requires *Shh* protein for its activation. Cross-regulatory interactions between corresponding pairs of class I and class II genes determine the borders of the neural subgroups (reviewed in references 5, 27, and 38). Most of these homeobox genes contain an EH1 motif that is bound by the corepressors *Groucho*/*TLE*, which are expressed in the ventral neural tube at the time when neural patterning occurs (33). The presence of an EH1 motif in the *Sax2* gene indicates a possible function as a transcription repressor but does not exclude an additional activator function. Homeobox genes can play a dual role as repressors or activators of transcription of their target genes depending on their interaction with specific cofactors (reviewed in reference 28).

## ACKNOWLEDGMENTS

We thank Carla Tribioli for providing the *IRESlacZ*/*floxedneo* cassette.

This work was supported in part by grants AR46471 and DE13741 from the NIH to T.L.

## REFERENCES

1. **Agarwala, S., and C. W. Ragsdale.** 2002. A role for midbrain arcs in nucleogenesis. Development **129:**5779–5788.
2. **Arber, S., B. Han, M. Mendelsohn, M. Smith, T. M. Jessell, and S. Sockanathan.** 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron **23:**659–674.
3. **Backman, C., T. Perlmann, A. Wallen, B. J. Hoffer, and M. Morales.** 1999. A selective group of dopaminergic neurons express Nurr1 in the adult mouse brain. Brain Res. **851:**125–132.
4. **Bengel, D., O. Johren, A. M. Andrews, A. Heils, R. Mossner, G. L. Sanvitto, J. M. Saavedra, K. P. Lesch, and D. L. Murphy.** 1997. Cellular localization and expression of the serotonin transporter in mouse brain. Brain Res. **778:**338–345.
5. **Briscoe, J., and J. Ericson.** 2001. Specification of neuronal fates in the ventral neural tube. Curr. Opin. Neurobiol. **11:**43–49.
6. **Cecchi, C.** 2002. Emx2: a gene responsible for cortical development, regionalization and area specification. Gene **291:**1–9.
7. **Chen, X., and T. Lufkin.** 1997. Linkage mapping of Sax2 to mouse chromosome 5. Mamm. Genome **8:**697–698.
8. **Chow, R. L., C. R. Altmann, R. A. Lang, and A. Hemmati-Brivanlou.** 1999. Pax6 induces ectopic eyes in a vertebrate. Development **126:**4213–4222.
9. **Chow, R. L., G. D. Roux, M. Roghani, M. A. Palmer, D. B. Rifkin, D. A. Moscatelli, and R. A. Lang.** 1995. FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. Development **121:**4383–4393.
10. **Danilov, V., M. Blum, A. Schweickert, M. Campione, and H. Steinbeisser.** 1998. Negative autoregulation of the organizer-specific homeobox gene goosecoid. J. Biol. Chem. **273:**627–635.
11. **Dohrmann, C., N. Azpiazu, and M. Frasch.** 1990. A new Drosophila homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. Genes Dev. **4:**2098–2111.
12. **Ericson, J., P. Rashbass, A. Schedl, S. Brenner-Morton, A. Kawakami, V. van Heyningen, T. M. Jessell, and J. Briscoe.** 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell **90:**169–180.
13. **Faber, S. C., M. L. Robinson, H. P. Makarenkova, and R. A. Lang.** 2002. Bmp signaling is required for development of primary lens fiber cells. Development **129:**3727–3737.
14. **Frasch, M., X. Chen, and T. Lufkin.** 1995. Evolutionary-conserved enhancers direct region-specific expression of the murine Hoxa-1 and Hoxa-2 loci in both mice and *Drosophila.* Development **121:**957–974.
15. **Furuta, Y., and B. L. Hogan.** 1998. BMP4 is essential for lens induction in the mouse embryo. Genes Dev. **12:**3764–3775.
16. **Gingrich, J. A., and R. Hen.** 2001. Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. Psychopharmacology (Berlin) **155:**1–10.
17. **Gonzalez, G., L. Puelles, and L. Medina.** 2002. Organization of the mouse dorsal thalamus based on topology, calretinin immunostaining, and gene expression. Brain Res. Bull. **57:**439–442.
18. **Hahm, S., T. M. Mizuno, T. J. Wu, J. P. Wisor, C. A. Priest, C. A. Kozak, C. N. Boozer, B. Peng, R. C. McEvoy, P. Good, K. A. Kelley, J. S. Takahashi, J. E. Pintar, J. L. Roberts, C. V. Mobbs, and S. R. Salton.** 1999. Targeted deletion of the Vgf gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance. Neuron **23:**537–548.
19. **Hawkins, N. C., and J. D. McGhee.** 1990. Homeobox containing genes in the nematode *Caenorhabditis elegans.* Nucleic Acids Res. **18:**6101–6106.
20. **Holland, P. W.** 2001. Beyond the Hox: how widespread is homeobox gene clustering? J. Anat. **199:**13–23.
21. **Hynes, M., and A. Rosenthal.** 1999. Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. Curr. Opin. Neurobiol. **9:**26–36.
22. **Hynes, M., W. Ye, K. Wang, D. Stone, M. Murone, F. Sauvage, and A. Rosenthal.** 2000. The seven-transmembrane receptor smoothened cell-autonomously induces multiple ventral cell types. Nat. Neurosci. **3:**41–46.
23. **Iijima, N., M. Tanaka, S. Mitsui, Y. Yamamura, N. Yamaguchi, and Y. Ibata.** 1999. Expression of a serine protease (motopsin PRSS12) mRNA in the mouse brain: in situ hybridization histochemical study. Brain Res. Mol. Brain Res. **66:**141–149.
24. **Jessell, T. M.** 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. **1:**20–29.
25. **Knirr, S., N. Azpiazu, and M. Frasch.** 1999. The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning. Development **126:**4525–4535.
26. **Kurata, T., and N. Ueno.** 2003. *Xenopus* Nbx, a novel NK-1 related gene essential for neural crest formation. Dev Biol. **257:**30–40.
27. **Lee, S. K., and S. L. Pfaff.** 2001. Transcriptional networks regulating neuronal identity in the developing spinal cord. Nat. Neurosci. **4**(Suppl 1A)**:**1183–1191.
28. **Lufkin, T.** 2000. Developmental control by Hox transcriptional regulators and their cofactors, p. 215–235. *In* J. Locker (ed.), Transcription factors. Bios Scientific Publishers, Oxford, United Kingdom.
29. **Manzanares, M., S. Bel-Vialar, L. Ariza-McNaughton, E. Ferretti, H. Marshall, M. M. Maconochie, F. Blasi, and R. Krumlauf.** 2001. Independent regulation of initiation and maintenance phases of Hoxa3 expression in the vertebrate hindbrain involve auto- and cross-regulatory mechanisms. Development **128:**3595–3607.
30. **Matise, M. P., and A. L. Joyner.** 1997. Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. J. Neurosci. **17:**7805–7816.
31. **Matsushita, N., H. Okada, Y. Yasoshima, K. Takahashi, K. Kiuchi, and K. Kobayashi.** 2002. Dynamics of tyrosine hydroxylase promoter activity during midbrain dopaminergic neuron development. J. Neurochem. **82:**295–304.
32. **Moretti, P., P. Simmons, P. Thomas, D. Haylock, P. Rathjen, M. Vadas, and R. D'Andrea.** 1994. Identification of homeobox genes expressed in human haemopoietic progenitor cells. Gene **144:**213–219.
33. **Muhr, J., E. Anderson, M. Persson, T. M. Jessell, and J. Ericson.** 2001.

Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell **104:**861–873.
34. **Nagy, A., C. Moens, E. Ivanyi, J. Pawling, M. Gertsenstein, A. K. Hadjantonakis, M. Pirity, and J. Rossant.** 1998. Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. Curr. Biol. **8:**661–664.
35. **Oliver, G., M. Vispo, A. Mailhos, C. Martinez, B. Sosa-Pineda, W. Fielitz, and R. Ehrlich.** 1992. Homeoboxes in flatworms. Gene **121:**337–342.
36. **Packer, A. I., D. A. Crotty, V. A. Elwell, and D. J. Wolgemuth.** 1998. Expression of the murine Hoxa4 gene requires both autoregulation and a conserved retinoic acid response element. Development **125:**1991–1998.
37. **Pfaar, H., A. von Holst, D. M. Vogt Weisenhorn, C. Brodski, J. Guimera, and W. Wurst.** 2002. mPet-1, a mouse ETS-domain transcription factor, is expressed in central serotonergic neurons. Dev. Genes Evol. **212:**43–46.
38. **Poh, A., A. Karunaratne, G. Kolle, N. Huang, E. Smith, J. Starkey, D. Wen, I. Wilson, T. Yamada, and M. Hargrave.** 2002. Patterning of the vertebrate ventral spinal cord. Int. J. Dev. Biol. **46:**597–608.
39. **Pratt, T., T. Vitalis, N. Warren, J. M. Edgar, J. O. Mason, and D. J. Price.** 2000. A role for Pax6 in the normal development of dorsal thalamus and its cortical connections. Development **127:**5167–5178.
40. **Protonotariou, E., A. Malamitsi-Puchner, G. Giannaki, D. Rizos, I. Phocas, and A. Sarandakou.** 1999. Patterns of inflammatory cytokine serum concentrations during the perinatal period. Early Hum. Dev. **56:**31–38.
41. **Rangini, Z., A. Frumkin, G. Shani, M. Guttmann, H. Eyal-Giladi, Y. Gruenbaum, and A. Fainsod.** 1989. The chicken homeo box genes CHox1 and CHox3: cloning, sequencing and expression during embryogenesis. Gene **76:**61–74.
42. **Rezgaoui, M., G. Hermey, I. B. Riedel, W. Hampe, H. C. Schaller, and I. Hermans-Borgmeyer.** 2001. Identification of SorCS2, a novel member of the VPS10 domain containing receptor family, prominently expressed in the developing mouse brain. Mech. Dev. **100:**335–338.
43. **Rhinn, M., and M. Brand.** 2001. The midbrain-hindbrain boundary organizer. Curr. Opin. Neurobiol. **11:**34–42.
44. **Rovescalli, A. C., M. Cinquanta, J. Ferrante, C. A. Kozak, and M. Nirenberg.** 2000. The mouse Nkx-1.2 homeobox gene: alternative RNA splicing at canonical and noncanonical splice sites. Proc. Natl. Acad. Sci. USA **97:**1982–1987.
45. **Schubert, F. R., A. Fainsod, Y. Gruenbaum, and P. Gruss.** 1995. Expression of the novel murine homeobox gene Sax-1 in the developing nervous system. Mech. Dev. **51:**99–114.
46. **Seale, P., L. A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, and M. A. Rudnicki.** 2000. Pax7 is required for the specification of myogenic satellite cells. Cell **102:**777–786.
47. **Smidt, M. P., H. S. van Schaick, C. Lanctot, J. J. Tremblay, J. J. Cox, A. A. van der Kleij, G. Wolterink, J. Drouin, and J. P. Burbach.** 1997. A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. Proc. Natl. Acad. Sci. USA **94:**13305–13310.
48. **Smith, S. T., and J. B. Jaynes.** 1996. A conserved region of engrailed, shared among all en-, gsc-, Nk1-, Nk2- and msh-class homeoproteins, mediates active transcriptional repression in vivo. Development **122:**3141–3150.
49. **Sosic, D., J. A. Richardson, K. Yu, D. M. Ornitz, and E. N. Olson.** 2003. Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. Cell **112:**169–180.
50. **Thaler, J. P., S. K. Lee, L. W. Jurata, G. N. Gill, and S. L. Pfaff.** 2002. LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. Cell **110:**237–249.
51. **Tribioli, C., M. Frasch, and T. Lufkin.** 1997. Bapx1: an evolutionary conserved homologue of the *Drosophila* bagpipe homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. Mech. Dev. **65:**145–162.
52. **Tribioli, C., and T. Lufkin.** 1999. The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. Development **126:**5699–5711.
53. **Wang, W., P. Lo, M. Frasch, and T. Lufkin.** 2000. Hmx: an evolutionary conserved homeobox gene family expressed in the developing nervous system in mice and *Drosophila.* Mech. Dev. **99:**123–137.
54. **Wurst, W., and L. Bally-Culf.** 2001. Neural plate patterning: upstream and downstream of the isthmic organizer. Nat. Rev. Neurosci. **2:**99–108.
55. **Xu, L., P. A. Overbeek, and L. W. Reneker.** 2002. Systematic analysis of E-, N- and P-cadherin expression in mouse eye development. Exp. Eye Res. **74:**753–760.
56. **Zhou, Y., B. C. Xu, H. G. Maheshwari, L. He, M. Reed, M. Lozykowski, S. Okada, L. Cataldo, K. Coschigamo, T. E. Wagner, G. Baumann, and J. J. Kopchick.** 1997. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proc. Natl. Acad. Sci. USA **94:**13215–13220.