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Expression of the novel murine homeobox gene *Sax-1* in the developing nervous system

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Abstract

We have isolated the novel murine *Sax-1* gene, a member of the NK-1 class of homeobox genes, and report its expression pattern in the developing central nervous system (CNS) in comparison to two other homeobox genes, *Evx-1* and *Pax-6*. *Sax-1* was found to be transiently expressed in the developing posterior CNS. First seen in the ectoderm lateral to the primitive streak, the signal later encompassed the neural plate. Posteriorly, the expression domain overlapped with the *Evx-1* expression in the streak, while anteriorly it was delimited by the *Pax-6* signal in the neural tube. This early phase of *Sax-1* expression suggests a role during the early determinative events in the formation of hindbrain and spinal cord. In a second phase starting at day 9.5 pc, *Sax-1* was expressed in distinct areas of spinal cord, hindbrain, midbrain and forebrain. Particularly strong signals were detected in rhombomere 1 and in the pretectum. In these areas, subsets of neurons may be marked and specified. In addition to the normal pattern of *Sax-1* during development, the expression in different mouse mutants was analysed. In *Brachyury curtailed* homozygotes, the expression of *Sax-1* was found to be reduced during neurulation and even lost at day 9.0 pc. Ventral shift and finally loss of the signal in the ventral spinal cord was observed in *Danforth's short tail* homozygotes.

Keywords: Neural induction; Homeobox; Spinal cord; P19 EC cells; *Brachyury curtailed*; *Danforth's short tail*; *Evx-1*; *Pax-6*

1. Introduction

The central nervous system (CNS) in vertebrates originates from a pseudostratified epithelium that through a series of proliferation and differentiation steps is turned into the complex structure of the adult brain and spinal cord. The first step in the formation of the CNS is the induction of ectodermal cells towards a neural fate (reviewed in Ruiz i Altaba, 1994). Extensive studies suggest neural induction to be composed of two processes: the activation of the cranial neuroectoderm and its subsequent transformation into more caudal fates (reviewed in Saxén, 1989). Studies on the molecular basis of neural induction support the idea that different mechanisms may underlie these two aspects of the induction: firstly, the so-far characterised candidate inducers lead to the formation of anterior CNS structures (reviewed in Ruiz i Altaba, 1994); secondly, regulatory genes expressed early enough to be involved in the response of the prospective neuroepithelial cells are restricted to either forebrain and midbrain or to hindbrain and spinal cord. Moreover, even the earliest markers for the anterior CNS identified up to now, such as *X-dll3* (Papalopulu and Kintner, 1993) or *Otx-2* (Simeone et al., 1993), may already be confined to specific regions, thus suggesting a close link of activation and regionalisation in the anterior CNS.

In contrast, in the chick, the *CHox3* gene (Rangini et al., 1989), recently renamed *cSax-1*, has been characterised, which is transiently expressed in the birth zone of the whole spinal cord regardless of the axial level (Spann et al., 1994). The gene is a member of the small NK-1 class of homeobox genes, which includes other representatives in chordates (Bober et al., 1994),

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platyhelminthes (Oliver et al., 1992), nemathelminthes (Hawkins and McGhee, 1990) and insects (Dohrmann et al., 1990; Kim and Nirenberg, 1989; Walldorf et al., 1989). The *Drosophila* representative *NK-1/S59* has been shown to be expressed, apart from subsets of muscle and midgut cells, during the formation of the fly CNS (Dohrmann et al., 1990). Its expression in distinct ganglion mother cells seems to justify the classification as a neural identity gene, although the final proof in the form of a mutation is still missing.

To further investigate this class of homeobox genes possibly involved in cell specification processes during the development of the nervous system, we have isolated the murine homologue of the chick *CHox3/cSax-1* gene. The expression pattern of the mouse *Sax-1* gene closely resembles that of *CHox3/cSax-1*. Transcripts were first detected at day 7.0 pc in the ectoderm lateral to the primitive streak. In comparison to the *Evx-1* pattern in the primitive streak, the *Sax-1* signal appeared slightly later and was located more anterior. The *Sax-1* staining later extended anteriorly, transiently labelling the developing posterior neural tube up to the *Pax-6* expression domain, i.e. the level of the last formed somite. The expression pattern of *Sax-1* suggests that the gene is active in all cells of the posterior CNS during their specification. In agreement with a possible role of the gene early during neural cell development, *Sax-1* expression was induced early during the differentiation of P19 EC cells into the neural lineage.

In addition, we have extended the expression analysis to later stages of development, when *Sax-1* is expressed in the differentiating CNS, particularly high in rhombomere 1 and in the pretectum. During this later phase of expression, *Sax-1* may play a role in spatial patterning or in the specification of subsets of neurons. To reveal possible mechanisms regulating the *Sax-1* expression, we analysed several mouse mutants for alterations in *Sax-1* expression.

2. Results

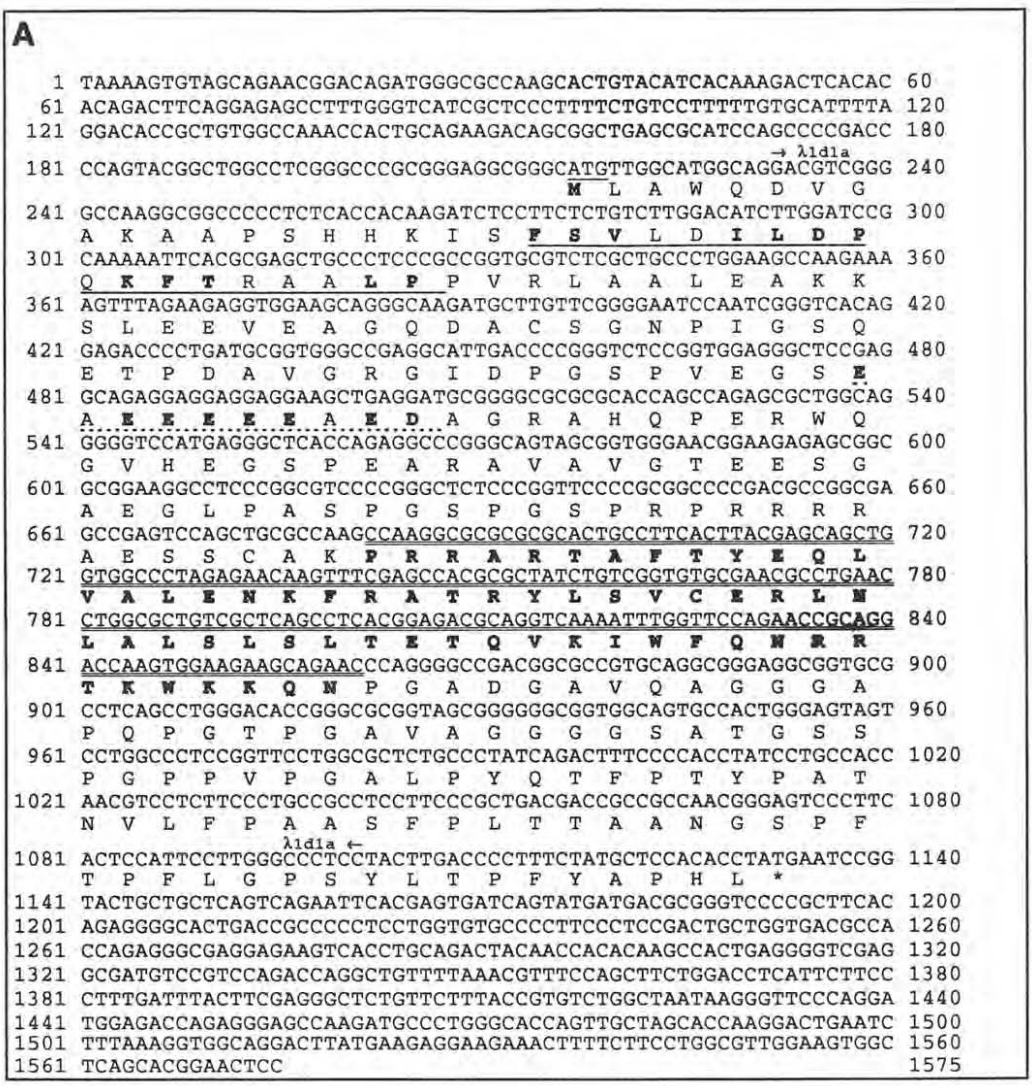
2.1. Isolation and characterisation of the mouse Sax-1 gene

A 76-bp homeobox fragment of the murine *Sax-1* gene was amplified by polymerase chain reaction (PCR) using degenerate primers specific for the NK-1 class of homeobox genes. After initial identification by hybridisation to the homeobox of the chicken *CHox-3/cSax-1* gene (Rangini et al., 1989) and further characterisation by sequencing, the fragment was used to screen a cDNA library obtained from mouse day 8.5 embryos (Fahrner et al., 1987). Two clones containing the mouse *Sax-1* homeobox were isolated, a 869-basepairs (bp) clone and a 1575-bp cDNA entirely enclosing the former. None of these clones showed a poly(A) stretch or a polyadenylation signal, suggesting that they may not represent full length transcripts. However, sequencing revealed a single open reading frame of 915 bp, encoding 305 amino acids (Fig. 1A). This putative open reading frame starts at position 217 of the longer cDNA with the only methionine codon in frame with the homeobox while its 3′ end is defined by a stop codon at position 1132. Thus, the longer cDNA clone probably harbours the entire coding region for the mouse *Sax-1* protein.

In the deduced protein sequence of mouse *Sax-1*, the homeodomain resides roughly in the centre. Upon closer inspection of the sequence, additional domains became evident (Fig. 1B): as a common feature of homeobox genes, basic amino acids accumulate around the N-terminus of the homeodomain. In addition, an acidic domain composed of eight glutamic or aspartic acid residues was found between amino acids 88 and 97. Towards the C-terminus, small uncharged amino acids like glycine, proline, serine, or alanine dominate. The comparison with the partial cSax-1 sequence reveals that the similarity between the two proteins extends in both directions outside the homeodomain. Even more informative is the look on the C-terminal third of the *Drosophila* NK-1 protein (Dohrmann et al., 1990), in which all the conspicuous domains of the mouse Sax-1 protein are present, suggesting a conserved function for these parts of the proteins. In addition, both proteins share a highly conserved domain of unknown function at the N-terminus of mouse Sax-1. However, the fly protein carries further domains like a PRD repeat not represented in the isolated mouse *Sax-1* transcript.

The comparison of homeobox sequences clearly characterises mouse *Sax-1* as a member of the NK-1 class of homeobox genes (Fig. 1C). The similarity to other members of this class ranges from 65% (*ceh-1*) to 84% (*CHox3/cSax-1*) on the nucleotide level, and from

Fig. 1. The murine *Sax-1* gene. (A) Sequence of the longer cDNA clone of the murine *Sax-1* gene. The deduced protein sequence of the longest open reading frame including the homeodomain (bold) is written underneath the nucleotide sequence. Also indicated are start and end of the second cDNA clone (arrows), a domain conserved between *Sax-1* and *NK-1/S59* (underlined), and a highly acidic domain (dotted line). The sequence has been deposited at the EMBL library (accession number X75384). (B) Schematic representation of conspicuous domains in the deduced mouse *Sax-1* protein sequence. Domains were identified due to unusual amino acid composition and due to conservation between mouse *Sax-1* and the *Drosophila NK-1/S59* protein. (C) Comparison of the homeodomains of the NK-1 class and other homeobox genes. Dashes stand for residues identical to the mouse *Sax-1* sequence. The highest similarity is found for the chick *Sax-1* gene, but the murine *Nkx-1.1* gene is also highly related. References are indicated in the text.


A
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61 ACAGACTTCAGGAGAGCCTTTGGGTCATCGCTCCCTTTTCTGTCCTTTTTGTGCATTTTA 120
121 GGACACCGCTGTGGCCAAACCACTGCAGAAGACAGCGGCTGAGCGCATCCAGCCCCGACC 180
→ λ1d1a
181 CCAGTACGGCTGGCCTCGGGCCCGCGGGAGGCGGGCATGTTGGCATGGCAGGACGTCGGG 240
M L A W Q D V G
241 GCCAAGGCGGCCCCCTCTCACCACAAGATCTCCTTCTCTGTCTTGGACATCTTGGATCCG 300
A K A A P S H H K I S F S V L D I L D P
301 CAAAAATTCACGCGAGCTGCCCTCCCGCCGGTGCGTCTCGCTGCCCTGGAAGCCAAGAAA 360
Q K F T R A A L P P V R L A A L E A K K
361 AGTTTAGAAGAGGTGGAAGCAGGGCAAGATGCTTGTTCGGGGAATCCAATCGGGTCACAG 420
S L E E V E A G Q D A C S G N P I G S Q
421 GAGACCCCTGATGCGGTGGGCCGAGGCATTGACCCCGGGTCTCCGGTGGAGGGCTCCGAG 480
E T P D A V G R G I D P G S P V E G S E
481 GCAGAGGAGGAGGAGGAAGCTGAGGATGCGGGGCGCGCGCACCAGCCAGAGCGCTGGCAG 540
A E E E E E E A E D A G R A H Q P E R W Q
541 GGGGTCCATGAGGGCTCACCAGAGGCCCGGGCAGTAGCGGTGGGAACGGAAGAGAGCGGC 600
G V H E G S P E A R A V A V G T E E S G
601 GCGGAAGGCCTCCCGGCGTCCCCGGGCTCTCCCGGTTCCCCGCGGCCCCGACGCCGGCGA 660
A E G L P A S P G S P G S P R P R R R R
661 GCCGAGTCCAGCTGCGCCAAGCCAAGGCGCGCGCGCACTGCCTTCACTTACGAGCAGCTG 720
A E S S C A K P R R A R T A F T Y E Q L
721 GTGGCCCTAGAGAACAAGTTTCGAGCCACGCGCTATCTGTCGGTGTGCGAACGCCTGAAC 780
V A L E N K F R A T R Y L S V C E R L N
781 CTGGCGCTGTCGCTCAGCCTCACGGAGACGCAGGTCAAAATTTGGTTCCAGAACCGCAGG 840
L A L S L S L T E T Q V K I W F Q N R R
841 ACCAAGTGGAAGAAGCAGAACCCAGGGGCCGACGGCGCCGTGCAGGCGGGAGGCGGTGCG 900
T K W K K Q N P G A D G A V Q A G G G A
901 CCTCAGCCTGGGACACCGGGCGCGGTAGCGGGGGGCGGTGGCAGTGCCACTGGGAGTAGT 960
P Q P G T P G A V A G G G G S A T G S S
961 CCTGGCCCTCCGGTTCCTGGCGCTCTGCCCTATCAGACTTTCCCCACCTATCCTGCCACC 1020
P G P P V P G A L P Y Q T F P T Y P A T
1021 AACGTCCTCTTCCCTGCCGCCTCCTTCCCGCTGACGACCGCCGCCAACGGGAGTCCCTTC 1080
N V L F P A A S F P L T T A A N G S P F
λ1d1a ←
1081 ACTCCATTCCTTGGGCCCTCCTACTTGACCCCTTTCTATGCTCCACACCTATGAATCCGG 1140
T P F L G P S Y L T P F Y A P H L *
1141 TACTGCTGCTCAGTCAGAATTCACGAGTGATCAGTATGATGACGCGGGTCCCCGCTTCAC 1200
1201 AGAGGGGCACTGACCGCCCCCTCCTGGTGTGCCCCTTCCCTCCGACTGCTGGTGACGCCA 1260
1261 CCAGAGGGCGAGGAGAAGTCACCTGCAGACTACAACCACACAAGCCACTGAGGGGTCGAG 1320
1321 GCGATGTCCGTCCAGACCAGGCTGTTTTAAACGTTTCCAGCTTCTGGACCTCATTCTTCC 1380
1381 CTTTGATTTACTTCGAGGGCTCTGTTCTTTACCGTGTCTGGCTAATAAGGGTTCCCAGGA 1440
1441 TGGAGACCAGAGGGAGCCAAGATGCCCTGGGCACCAGTTGCTAGCACCAAGGACTGAATC 1500
1501 TTTAAAGGTGGCAGGACTTATGAAGAGGAAGAAACTTTTCTTCCTGGCGTTGGAAGTGGC 1560
1561 TCAGCACGGAACTCC 1575
B
1
156
215
305
67% identical to NK-1/S59
80% acidic
83/67% basic
homeo domain
61% Gly/Pro
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mouse Sax-1 PRRARTAFTYEQLVALENKFRATRYLSVCERLNLALSLSLTETQVKIWFQNRRTKWKKQN
CHox3/cSax-1 ----------H
Nkx-1.1 ----------K----------G----------
Salbox-1 .....----------KC----------
Salbox-2 .....----------KS------SA----PV----------
NK-1/S59 ----------S-----KT----------
H40 A----------KT----------
ceh1 M----------KTS-----V------IQ-Q-S----------H-
Eghbx1 R----------T-----QS------Y---------N----------
msh N-KP--P--TQ--LS--K---EKQ---IA--AEFSS--R----------A-A-RLQ

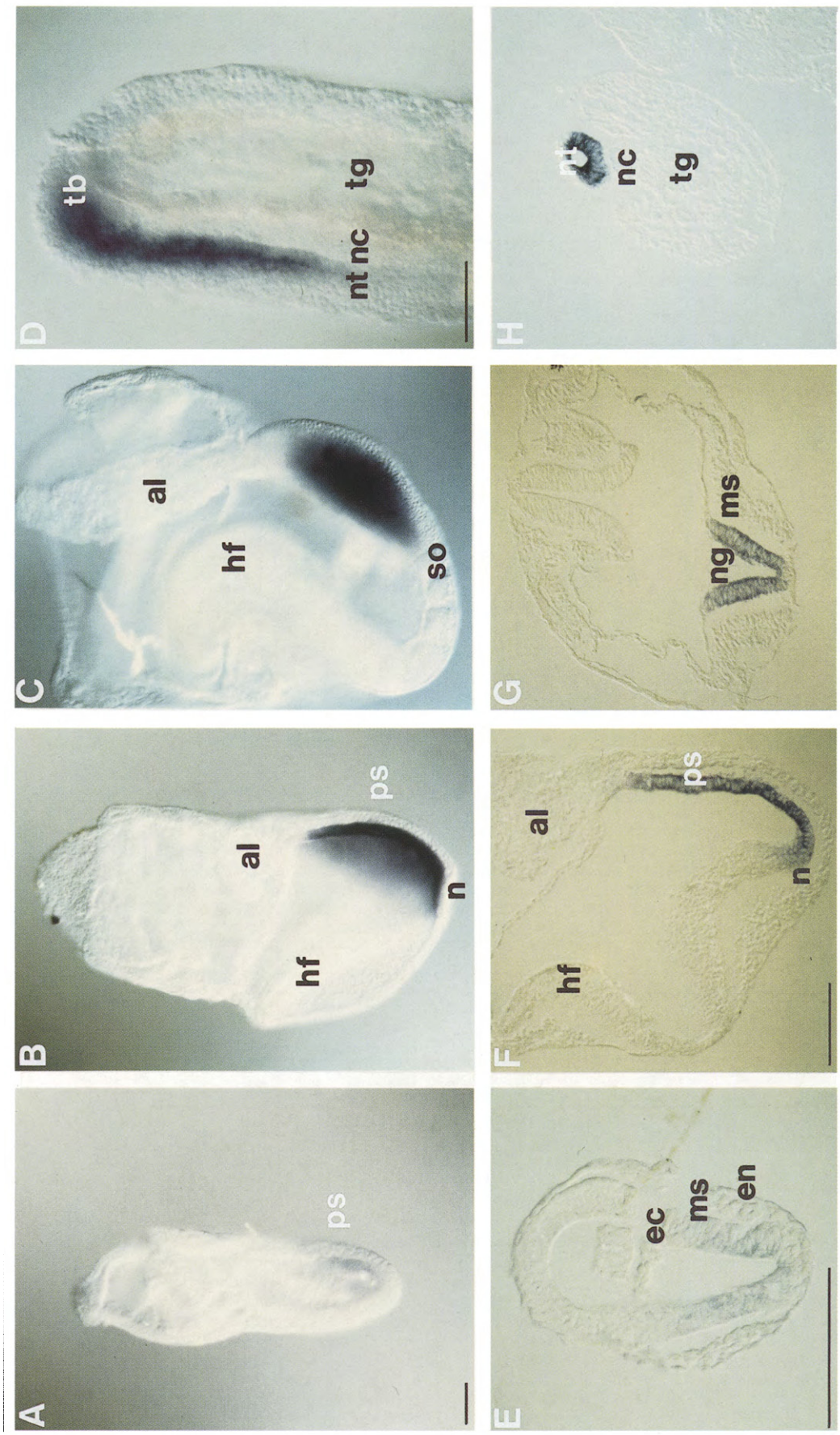


Fig. 2. Expression of *Sax-1* during primary and secondary neurulation. (A) Lateral view of and cross section (E) through a mid-streak mouse embryo at day 7.0 pc. *Sax-1* transcripts in the ectoderm lateral to the streak appear as a faint blue staining. Later, the *Sax-1* signal extends continuously from the primitive streak ectoderm into the neural groove anterior to the node, as shown in lateral view (B) and sagittal section (F) of an early headfold stage embryo at day 7.5 pc. Finally, the *Sax-1* expression domain resides mainly in the forming neuroepithelium, anteriorly ending at the level of the last formed somite, visible in lateral view (C) and cross section (G) through a 5-somite embryo at day 8.0 pc. During secondary neurulation, *Sax-1* likewise is expressed in the dorsal part of the tail bud, extending into the neural tube, exemplified by the lateral view (D) and the cross section (H) through the caudal end of a day 10.5 pc embryo. al, allantois; ec, ectoderm; en, endoderm; hf, headfold; ms, mesoderm; n, node; nc, notochord; ng, neural groove; nt, neural tube; ps, primitive streak; so, last-formed somite; tg, tail gut.

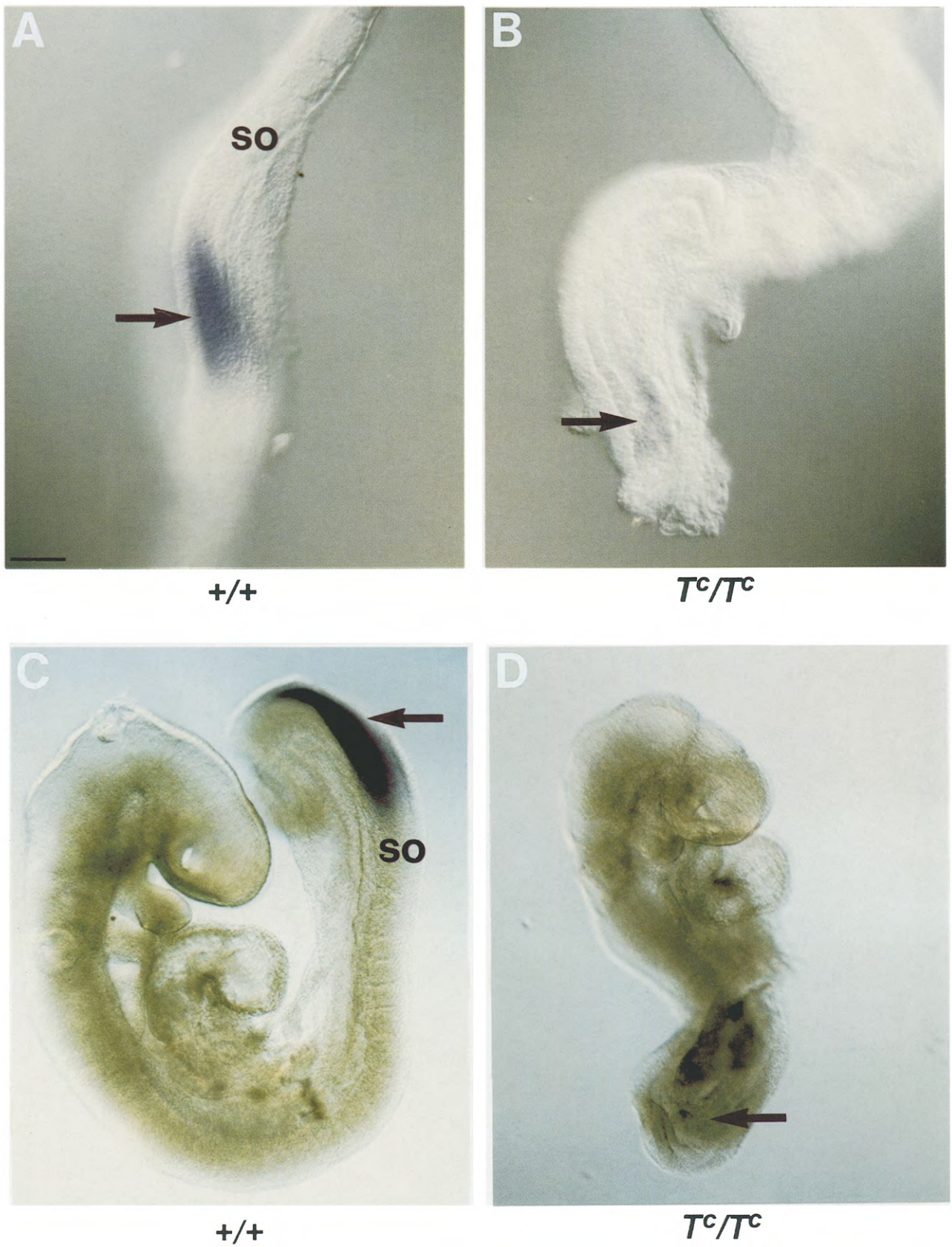
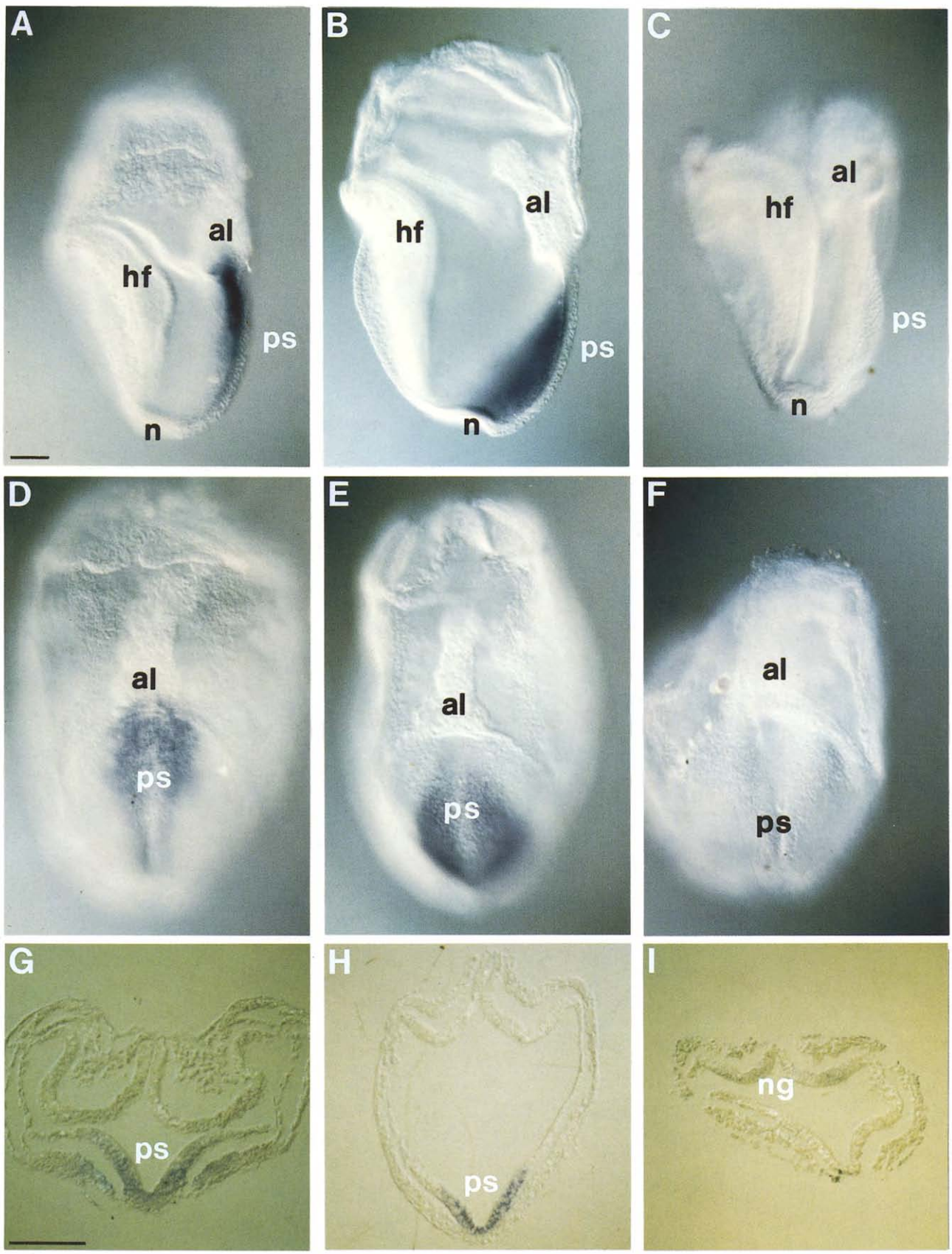


Fig. 3. Loss of *Sax-1* expression in *Brachyury curtailed* (T^c/T^c) embryos. (A) Wildtype and (B) T^c/T^c embryo at day 8.5 pc, showing the *Sax-1* expression in primitive streak and neural groove (arrows). In the T^c/T^c embryo the neural tube appears heavily kinked, the *Sax-1* signal is already weakened. One day later, *Sax-1* is strongly expressed around the posterior neuropore in the wildtype embryo (C, arrow), whereas in the homozygous mutant embryo the signal is no longer detectable (D).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Sax-1
Pax-6

87 to 98% on the amino acid level, indicating the high conservation of the protein sequence. The most closely related homeobox class represented by the *Drosophila msh* gene (Gehring, 1987) exhibits only 60% similarity on the nucleotide and 58% on the amino acid level showing that both classes are clearly distinct.

Within the vertebrate NK-1 class, the homeodomains of *CHox3/cSax-1* and mouse *Sax-1* are identical except for the 60th position, suggesting the two genes represent homologues. To underline this close evolutionary relationship, we renamed our previously called *Nkx1.1* gene (Yamada et al., 1994) mouse *Sax-1*. Recently, another member of the NK-1 homeobox class has been identified in the mouse, also called *Nkx-1.1* (Bober et al., 1994). The homeodomain of this gene differs in two positions from the mouse *Sax-1* sequence, suggesting both mouse genes to be paralogues.

2.2. Formation of the posterior CNS: the first phase of Sax-1 expression

The temporal and spatial pattern of *Sax-1* expression was studied by whole mount in situ hybridisation on postimplantation mouse embryos starting with day 6.5 pc. *Sax-1* transcripts were first detected at day 7.0 pc in the mid-streak egg cylinder (Fig. 2A). The expression appeared restricted to a broad pairwise stripe in the ectoderm aligning the primitive streak (Fig. 2E). Anteriorly, the signal was delimited by the end of the primitive streak, while posteriorly it faded out before reaching the caudal limit. The signal appeared strongest just lateral to the streak, waning more lateral and in the midline.

Up to the headfold stage at day 7.5 pc, the *Sax-1* staining increased, still confined to the ectodermal layer along the primitive streak and posterior to the node. Starting with day 7.5 pc, however, roughly simultaneous with the onset of somitogenesis, the *Sax-1* signal extended anterior to the node into the neural plate (Figs. 2B,F and 4B). Cross sections through the primitive streak at this stage revealed expression in the columnar ectodermal cells while the flat cells located laterally were negative (Fig. 4H). Thus, also the lateral margin of *Sax-1* expression appeared sharpened now, suggesting a more defined delimitation between neuro- and surface ectoderm.

In the following hours, the major expression domain shifted from the primitve streak area to the developing neuroepithelium (Fig. 2C): at day 8.0 pc, the strongest signal was confined to the open neural groove. In contrast, the prospective epidermal cells were negative and the neural folds in between were generally weakly labelled. In addition, the cells in the ventral midline of the neuroepithelium, adjacent to the notochord showed less intense staining (Fig. 2G). Cross sections through the closing neural tube at day 9.0 pc similarly demonstrated the *Sax-1* expression in the recently formed neuroectoderm. In this area, the whole neuroepithelium was stained, leaving out only the fusing neural folds and the epidermis above (not shown). In all stages, the *Sax-1* signal decreased at about the level of the last formed somite, illustrating that *Sax-1* transcripts are restricted to the site of neuroectoderm formation at the posterior pole of the embryo (Figs. 2C, 3A and C).

As the remnants of the primitive streak turn into the tail bud around day 10.0 pc and secondary neurulation starts, *Sax-1* is expressed in the dorsal tail bud mesenchyme (Fig. 2D). The signal extended continuously into the neural tube (Fig. 2H), anteriorly fading out at the level of the recently formed somite. Thus, despite the different morphology of primary and secondary neurulation, *Sax-1* appeared transiently expressed in the developing trunk neuroepithelium in a similar manner during both processes.

2.3. Brachyury: loss of Sax-1 expression

To study the effect of impaired mesoderm development on the expression of *Sax-1* as an early neuroectoderm-specific marker, we characterised the expression pattern of *Sax-1* in the *T* gene (Herrmann et al., 1990) mutant allele *Brachyury curtailed* (T^c) (Fig. 3). The expression level of *Sax-1* appeared to be directly correlated to the extent of malformations at the caudal pole of the embryos. While at day 7.5–8.0 pc, the *Sax-1* signal appeared just slightly reduced compared to the wildtype (not shown), the *Sax-1* expression at day 8.5 pc was already strongly reduced (Fig. 3B). At this stage, the homozygotes are grossly malformed in the posterior part. The neural tube is heavily kinked, has morphologically defined somites and the allantois are lacking. Half a day later, *Sax-1* transcripts vanished completely (Fig. 3D). These findings suggest that mesoderm formation and *T* gene function are not required for *Sax-1* induction. However, the impaired posterior body formation in T^c/T^c embryos abolishes *Sax-1* expression.

Fig. 4. Comparison of *Evx-1*, *Sax-1* and *Pax-6* expression in headfold stage embryos. (A–C) lateral views, (D-F) ventral views on the primitive streak and (G–I) cross sections of headfold stage embryos. *Evx-1* is expressed in all three germ layers throughout the whole length of the streak from the base of the allantois to the node, predominantly however in the posterior part. The *Sax-1* domain does not extend as far caudal as the *Evx-1* signal, but spreads out more lateral and extends anterior to the node into the neural groove. Only ectodermal cells are labelled. *Pax-6* at this stage just starts to be expressed weakly in the neural groove, directly anterior to the cranial limit of the *Sax-1* signal.

2.4. Potential regulators: comparison to Evx-1 and Pax-6

The expression profile of *Sax-1* closely resembles the pattern of another homeobox gene, *Evx-1* (Bastian and Gruss, 1990; Dush and Martin, 1992). In comparative whole mount analyses, *Evx-1* expression could be detected in the early-streak egg cylinder slightly preceeding the *Sax-1* activity (not shown). The transcripts of both genes were restricted to the posterior aspect of the embryo (Fig. 4A and B). However, while the *Sax-1* signal was found only in the ectodermal layer (Fig. 4H), *Evx-1* was expressed in all three germ layers (Fig. 4G). Furthermore, *Evx-1* showed the highest expression at the posterior end of the primitive streak and in the base of the allantois (Fig. 4D), where *Sax-1* was not expressed at all (Fig. 4E). In a narrow zone, the expression domains of both genes overlapped, opening up the possibility of some regulatory interaction.

Another gene known to be expressed only in ectodermal derivatives and largely limited to the CNS is the paired box containing gene *Pax-6* (Walther and Gruss, 1991). *Pax-6* transcripts were first detected at day 7.75 pc, approximately simultaneous with the onset of somitogenesis, just anterior to the *Sax-1* domain in the prospective spinal cord (Fig. 4C and I). During the following hours, as the *Sax-1* signal was retained in the region of neurogenesis at the posterior pole of the embryo, *Pax-6* occupied the newly added spinal cord tissues. To investigate whether *Pax-6* may be involved in the anterior delimitation of *Sax-1* transcription, the expression pattern of *Sax-1* in *Small eye* (*Sey*) embryos carrying a mutation in the *Pax-6* gene was analysed at day 9.5 pc. Among 14 embryos from two heterozygous matings, no alterations in the *Sax-1* staining were observed (not shown). Therefore, *Pax-6* appears not to be required to downregulate *Sax-1* activity during spinal cord maturation. Conversely, an activating capacity of *Sax-1* on the *Pax-6* gene cannot be excluded.

2.5. P19 EC cells: induction of Sax-1 expression upon differentiation

The expression of *Sax-1* in the developing posterior CNS suggests a function during the determination of ectodermal cells towards the neural fate. To test the mouse *Sax-1* gene for induction and repression during the course of neural cell differentiation, embryonic carcinoma (EC) cells served as simplified model system as P19 EC cells can be triggered to develop into neural cells by aggregation in culture medium containing retinoic acid (Jones-Villeneuve et al., 1982).

In undifferentiated P19 cells, *Sax-1* mRNA could not be detected by Northern blot analysis (Fig. 5). In contrast, *Sax-1* transcription was rapidly induced during neuronal differentiation: in EC cells aggregated for 2 days in the presence of retinoic acid, transcripts of 1.6, 1.9, 2.8, 3.9 and 6 kb were visible, the major band represented by the 3.9-kb molecules. However, morphologically these cells still appeared just as simple aggregates without neuronal features. In the following days of culture, the cells began to form neuronal processes and the amount of *Sax-1* transcripts declined. Finally, when after 10 days of differentiation neurons had accumulated, *Sax-1* transcripts were only barely

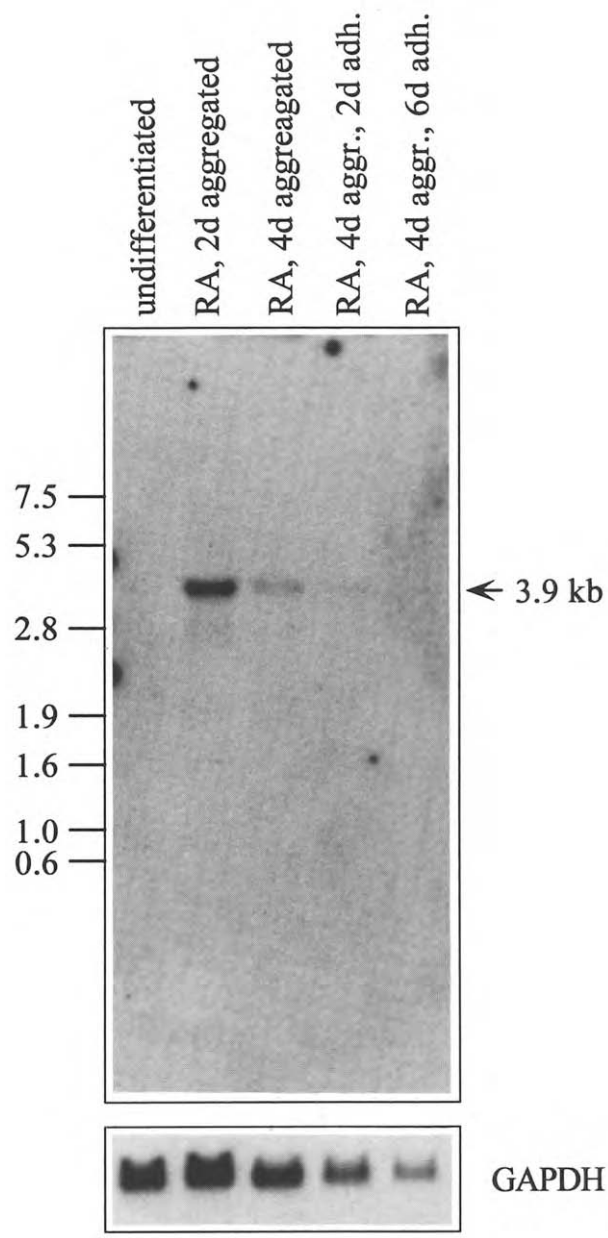

Fig. 5. RNA expression analysis in P19 EC cells. (A) P19 EC cells were differentiated into neural cells by aggregating them for 4 days in the presence of 3×10^{-7} M retinoic acid. Total RNA was prepared at 2, 4, 6 and 10 days after adding retinoic acid and hybridised to a *Sax-1* cDNA probe. No *Sax-1* transcripts are detectable in undifferentiated P19 EC cells. Already after 2 days of aggregation, several bands appear that decrease during the following days. (B) As a control for the total amount of RNA loaded, the same filter was hybridised to a probe for GAPDH.

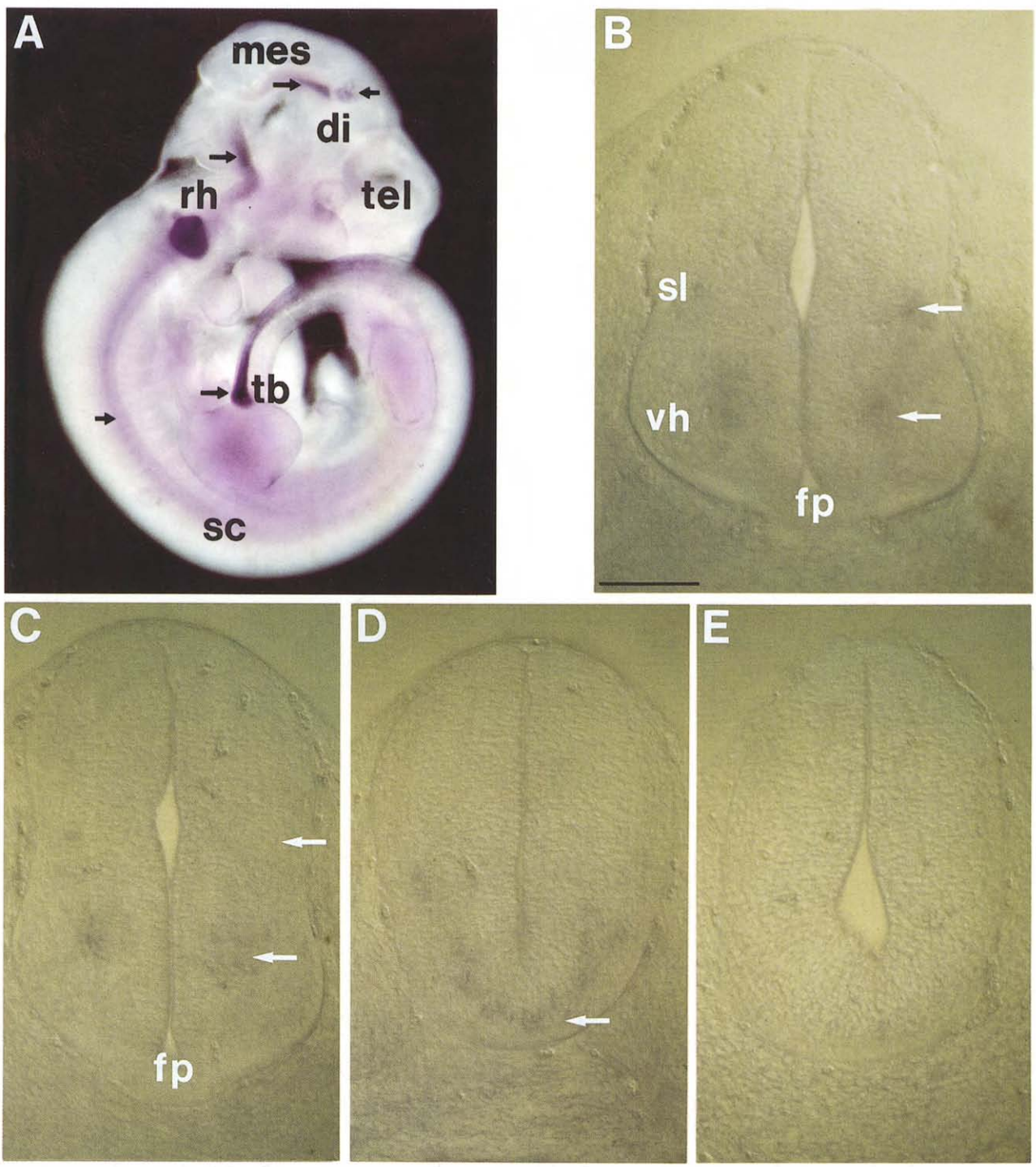

Fig. 6. Late phase of *Sax-1* expression in wildtype and *Danforth's short tail* (*Sd/Sd*) embryos. (A) Lateral view of a wildtype day 11 embryo. Apart from the tail bud, *Sax-1* is expressed in all three primary brain vesicles and in the spinal cord. In the spinal cord, the signal consists of two bilateral, longitudinal stripes. In cross sections at the cervical level (B), they appear in the ventrolateral part of the neural tube, medially and dorsally adjacent to the ventral horns (arrows). Cross sections through a *Sd/Sd* embryo at day 11 pc (C–E) reveal the wildtype-like pattern in the thoracic part (C, arrows), the ventral fusion of the signal in the lumbar area (D, arrow) and the final premature loss of the *Sax-1* signal (E). di, diencephalon; fl, forelimb bud; fp, floor plate; hl, hindlimb bud; mes, mesencephalon; rh, rhombencephalon; sc, spinal cord; sl, sulcus limitans; tel, telencephalon; vh, ventral horn.

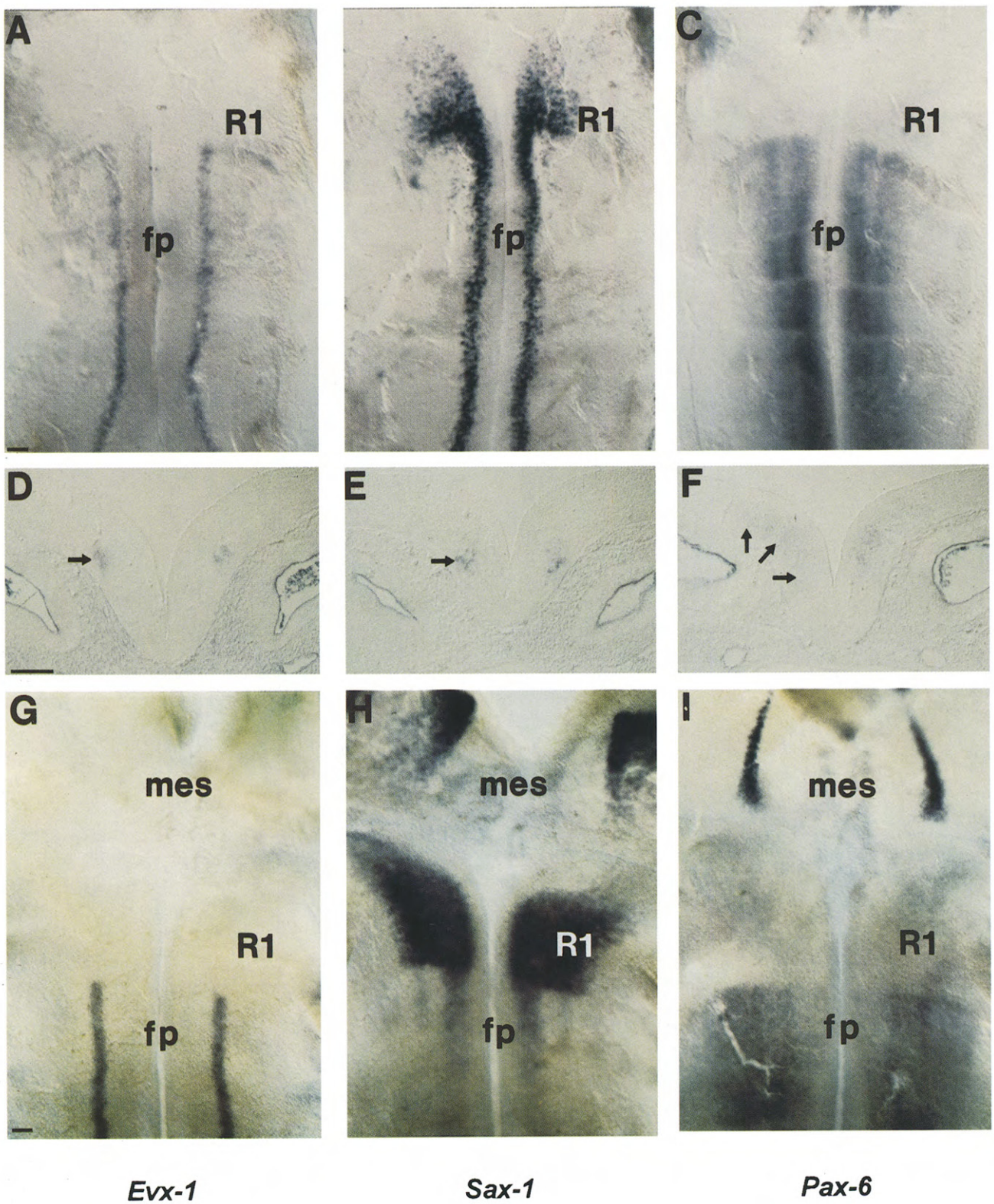

Fig. 7. Comparison of *Evx-1*, *Sax-1* and *Pax-6* expression in the hindbrain. (A–C) Ventral views on flat-mounted hindbrains at day 10.5 pc and (D–F) half-side cross sections at the level of the otic vesicle. (G–I) Ventral views on flat-mounted hindbrains at day 11 pc. *Evx-1* is completely and *Pax-6* mostly absent from rhombomere 1 in which *Sax-1* is strongly expressed throughout the basal plate. In the caudal rhombomeres, *Evx-1* and *Sax-1* are expressed in longitudinal stripes resembling the spinal cord signals. The *Sax-1* signal is located directly ventral to the *Evx-1* signal in the ventrolateral portion of the neural tube. *Pax-6* in contrast is expressed in a broad area in the medial basal plate. d, dorsal; fp, floor plate; mes, mesencephalon; R1, rhombomere 1; v, ventral.

detectable, suggesting that in vitro mouse *Sax-1* is an early marker for cells responding to neural induction.

2.6. Spinal cord and hindbrain: Sax-1 expression in the differentiating CNS

A second phase of *Sax-1* expression (Fig. 6) is initiated in the differentiating CNS between day 9.5 and day 10.0 pc. At day 9.5 pc, a few cells in the hindbrain started to express *Sax-1* (not shown). During the following hours, this signal strengthened and extended caudally into the spinal cord, reaching the level of the hindlimb buds at day 10.0 pc (not shown). At this stage, the expression in the spinal cord was limited to a continuous, pairwise stripe (Fig. 7B), which on cross sections was located in the lateral part of the ventricular zone adjacent to the ventral horns (not shown). From day 11.0 pc onwards, these stripes were accompanied by a second pair, located just ventral to the sulcus limitans (Fig. 6B). As both domains reside within the basal plate, this *Sax-1* expression may belong to the ventral program of hindbrain and spinal cord.

2.7. Danforth's short tail: ventral shift and loss of the Sax-1 signal in the spinal cord

To study whether the second phase of *Sax-1* expression in the spinal cord indeed belongs to the ventral program thus depending on the activity of the notochord, we analysed the *Sax-1* expression in *Danforth's short tail* mice. For the notochord mutant *Danforth's short tail* (*Sd*), distortions in the formation of motor and interneurons have been described in the caudal region, where the notochord due to degeneration is already missing during the initial dorsoventral patterning of the neural tube (Bovolenta and Dodd, 1991). Furthermore, alterations in the expression pattern of *Pax*-genes in the neural tube have been found (Dietrich et al., 1993; Phelps and Dressler, 1993). In the case of *Pax-3*, the ventrally shifted expression pattern suggests a dorsalisation of the ventral neural tube in regions initially lacking the notochord (Dietrich et al., 1993), as observed after notochord extirpation (Goulding et al., 1993).

In the cranial spinal cord of homozygous *Sd/Sd* embryos at day 11.0 pc, the signal was indistinguishable from the wildtype, appearing as continuous, pairwise stripes (Fig. 6C). However, while in the wildtype the signal could be followed caudal to the hindlimb bud, in *Sd/Sd* embryos the staining stopped abruptly in front of the hindlimb, indicating that the notochord is required for proper *Sax-1* expression. Cross sections revealed that before vanishing completely (Fig. 6E), the *Sax-1* signals first shifted ventrally to fuse in the ventral midline (Fig. 6D), instead of forming separate bilateral stripes. A similar ectopic location in the ventral midline has been reported for motor neurons in the equivalent region of *Sd/Sd* embryos (Bovolenta and Dodd, 1991), suggesting a link between *Sax-1* and ventral cell types.

2.8. Sax-1 in the hindbrain: the case of rhombomere 1

The ventral stripe of *Sax-1* expression seen in the spinal cord extends anteriorly into the hindbrain. Again, *Evx-1* exhibited a related expression pattern (Bastian and Gruss, 1990) between day 10.0–10.5 pc, forming a continuous pairwise stripe in the ventrolateral ventricular zone dorsally adjacent to the *Sax-1* positive cells (Figs. 7A, B, D and E). At day 11.0 pc, an additional broad *Evx-1* positive band traversed the posterior hindbrain and the spinal cord, opposite to the former and both *Sax-1* stripes confined to the alar plate.

In contrast to *Sax-1* and *Evx-1*, the *Pax-6* positive cells formed a broad, diffuse domain in the ventral ventricular zone excluding the floor plate (Fig. 7C, F and I). Also different from the former, *Pax-6* expression was reduced at the rhombomere boundaries. The pattern consequently appeared segmentally arranged in the hindbrain (Fig. 7C and I). However, the most obvious difference in the expression of the three genes is the lack of *Evx-1* and *Pax-6* transcripts in rhombomere 1 (Fig. 7A, G, C and I), where the *Sax-1* positive domain appeared strongly dorsally extended, encompassing the whole ventral portion of rhombomere 1 except the floor plate (Fig. 7B and H). At day 10.5 pc, the caudal boundary to rhombomere 2 and the ventral limit towards the floor plate of this *Sax-1* expression domain were already well defined, while the staining petered out cranially and dorsally. At day 11.0 pc however, when the *Sax-1* signal in rhombomere 1 was further enhanced, now also its posterior and anterior margins appeared sharpened (Fig. 7H).

2.9 Midbrain and forebrain: Sax-1 expression in comparison to Pax-6

Besides spinal cord and hindbrain, during midgestation *Sax-1* is also active in anterior brain areas. At day 10.0 pc, a distinct domain in the ventral portion of the anterior mesencephalon, the tegmentum, became apparent. Half a day later, cells at the caudal border of the diencephalon, the pretectum, started to express *Sax-1* (data not shown). As development progressed, the pattern in midbrain and forebrain diversified (Fig. 8): at day 11.5 pc, in the tegmentum two *Sax-1* positive zones emerged caudally from a common centre at the cranial margin of the mesencephalon (Fig. 8A). At the equivalent stage, *Pax-6* mRNA, in addition to the expression pattern described previously (Walther and Gruss, 1991), was found in a single stripe in the midbrain, possibly located between the two *Sax-1* domains (Fig. 8B). The *Pax-6* stripe originated more caudal compared to the *Sax-1* stripes and extended more caudal almost up to the midbrain/hindbrain boundary.

Anteriorly, *Sax-1* was found to be expressed predominantly in the pretectum (Figs. 8A and C). In the anterior pretectum similar to *Pax-6*, *Sax-1* transcripts were confined to single, dispersed cells (Figs. 8C and D).

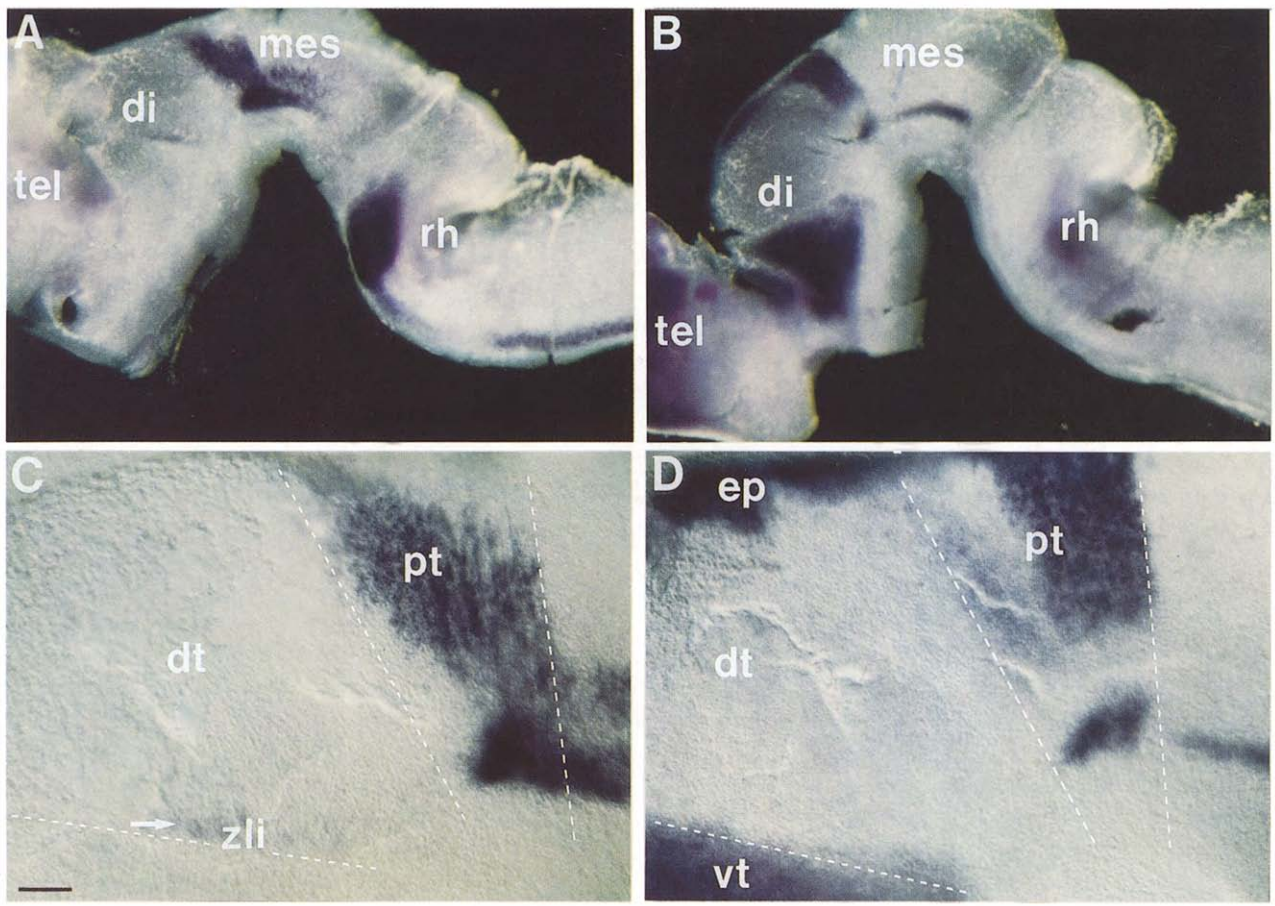

Fig. 8. Comparison of *Sax-1* and *Pax-6* expression in midbrain and forebrain. (A and B) Lateral views of dissected brains of day 11 pc mouse embryos and (C and D) details focussing on dorsal thalamus and pretectum. *Sax-1* is mainly expressed around the pretectum. From the basal plate of the prectectum, two stripes of expression extend through the midbrain. Within the pretectum, the posterior commissure and cells of the anterior pretectum are labelled. In addition, a thin array of cells between dorsal and ventral thalamus expressed *Sax-1* (arrow). The *Pax-6* expression in the midbrain is restricted to a single longitudinal stripe. In the diencephalon, strong *Pax-6* signals are seen in pretectum, epithalamus and ventral thalamus. In addition, the telencephalon is strongly stained. The *Pax-6* expression in the dorsal thalamus is weak, the hypothalamus appears negative. di, diencephalon; dt, dorsal thalamus; ep, epithalamus; mes, mesencephalon; pt, pretectum; rh, rhombencephalon; tel, telencephalon; zli, zona limitans intrathalamica.

In the alar plate of the posterior pretectum, a strong *Sax-1* staining was observed laterally along the axon tracts of the posterior commissure (Figs. 8A and C), while *Pax-6* was expressed more medially in the underlying cells of the ventricular zone (not shown). Moreover, transcripts of both genes were detected in a cluster of cells in the basal plate of the posterior pretectum, just anterior to the *Sax-1* positive cluster in the mesencephalon.

A further expression domain of *Sax-1* was located along the zona limitans intrathalamica, in a thin layer of cells between dorsal and ventral thalamus, thus separating domains of weak and strong *Pax-6* expression (Fig. 8C and D). Different from *Sax-1*, *Pax-6* was also strongly expressed in the whole epithalamus and in the telencephalon, as previously reported (Stoykova and Gruss, 1994; Walther and Gruss, 1991).

3. Discussion

The *Drosophila* gene *NK-1/S59* defines a small class of genes characterised by a highly conserved homeobox. Members of this class have been found in a variety of metazoan phyla ranging from flatworms to vertebrates. The expression patterns of *NK-1/S59* in *Drosophila* and *cSax-1* in chicken suggest a function in cell specification, particularly in the CNS. To get further insight into the role of NK-1 class genes in vertebrate development, we

have isolated the murine homologue of the *cSax-1* gene and have characterised the expression pattern of mouse *Sax-1* during the formation and the differentiation of the CNS.

Two cDNA clones were obtained, one of which represented the complete coding region of the corresponding mouse *Sax-1* transcript. Sequence comparisons revealed the highest similarity within and outside the homeodomain to the chicken gene *cSax-1*. However, since for *cSax-1* only a small portion of the sequence is available, the comparison is incomplete. Additional conserved domains therefore were determined by examining the *NK-1/S59* cDNA sequence (Dohrmann et al., 1990). Indeed, the search revealed common features, like a rather related stretch of amino acids at the amino terminus of mouse Sax-1, a cluster of basic amino acids in front of the homeodomain, and a high number of small uncharged amino acids at the carboxy terminus. Even more interesting, the mouse and *Drosophila* genes share a highly acidic domain in front of the homeodomain. Such 'acid blobs' have been shown to act as transcriptional activators in vitro (Ptashne, 1988). In contrast to NK-1/S59, our Sax-1 sequence lacks a PRD repeat. Since the Northern blot analysis revealed different transcripts for *Sax-1*, we cannot exclude the presence of different Sax-1 protein sequences, in some of which a PRD repeat may be found. Also, Southern blots showed at least one other related gene in the mouse genome, presumably *Nkx-1.1* (Bober et al., 1994), in which other features of the *Drosophila* gene may have been conserved.

3.1. Formation of the posterior CNS

The expression of *Sax-1* during embryogenesis was studied by RNA in situ hybridisation. Transcripts were found exclusively in the neuroectoderm, where two different phases of expression were distinguished.

During primary and secondary body formation, *Sax-1* expression was maintained in the birth zone of the CNS. As in the chick (not shown), transcripts of *Sax-1* were first detected in mid-streak embryos, in the ectoderm aligning the primitive streak. Fate maps describe this area as the primary source for posterior hindbrain and spinal cord (Lawson and Pedersen, 1992; Tam, 1989). Consequently, a few hours later the signal continuously extended anterior to the node into the neuroectoderm, where it remained cranially roughly delimited by the position of the last formed somite. As a result, all cells of the posterior CNS, from the posterior hindbrain to the tail, run through a phase of *Sax-1* expression during their differentiation.

The expression pattern of *Sax-1* differs in two aspects from that of known genes expressed early in the anterior CNS: its expression is transient and it is general for the whole posterior CNS rather than region-specific. Both differences may be two sides of one coin, the particularities of posterior CNS development. The fact that in the anterior CNS early expressed genes already occupy specific, spatially delimited regions suggests a close temporal or even causal connection of neural induction and anteroposterior regionalisation. In the posterior CNS, in contrast, regionalisation appears to be a relatively late event, conferred by e.g. *Krox-20* and the branchial *Hox* code in the hindbrain (reviewed in Krumlauf, 1993). For posterior CNS formation, therefore, a general activity responsible for the determination of the neuroepithelium prior to regionalisation should be proposed. *Sax-1*, being specifically expressed during the morphological transformation of simple ectodermal cells to the neuroepithelium, may be involved in this activity.

The caudal regression of the *Sax-1* signal during development and, therefore, a role in cell differentiation rather than axial patterning reminds of another homeobox gene, *Evx-1*. The *Evx-1* gene is transiently expressed during gastrulation in all three germ layers at the posterior end of the embryo (Bastian and Gruss, 1990; Dush and Martin, 1992). In *Xenopus*, the homologous gene has been demonstrated to be essential for the formation of the posterior body part: overexpression leads to premature initiation of posterior structures at the expense of anterior parts (Ruiz i Altaba and Melton, 1989), while the block of *Evx* function with antibodies abolishes posterior body formation (Ruiz i Altaba and Melton, 1991). Viewing the expression patterns of *Evx-1* and *Sax-1*, one might assume a series of determination events, reflected by the expression of these marker genes (Fig. 9A). *Evx-1* in this scenario would set the startpoint for posterior body formation, around the level of the first rhombomeres. The initiated process may afterwards be specified in the different germ layers by a set of secondary control genes, including *Sax-1* for the neuroectoderm. The activity of the secondary genes would guide the cells to a determined state, from which differentiation processes within each tissue start, exemplified by the *Pax-6* expression in the ventromedial portion of the neural tube. This hypothesis could be tested by repeating Ruiz i Altaba's experiments, checking for the expression of the *Xenopus Sax-1* gene. Indirect support already comes from the analysis of homozygous *Brachyury* mutant mice, in which the formation of the posterior body portion is heavily disturbed. Consistent with the possible function of these genes as regulators of posterior body formation, the expression of both *Evx-1* (Rashbass et al., 1994) and *Sax-1* is turned off in the mutant embryos. However, the lack of the signals may as well be explained by the trivial absence of expressing cells due to impaired body formation in the mutant.

Further studies on the *Sax-1* gene should be facilitated by the availability of P19 EC cells as an in vitro system. The expression of *Sax-1* during the differentia-

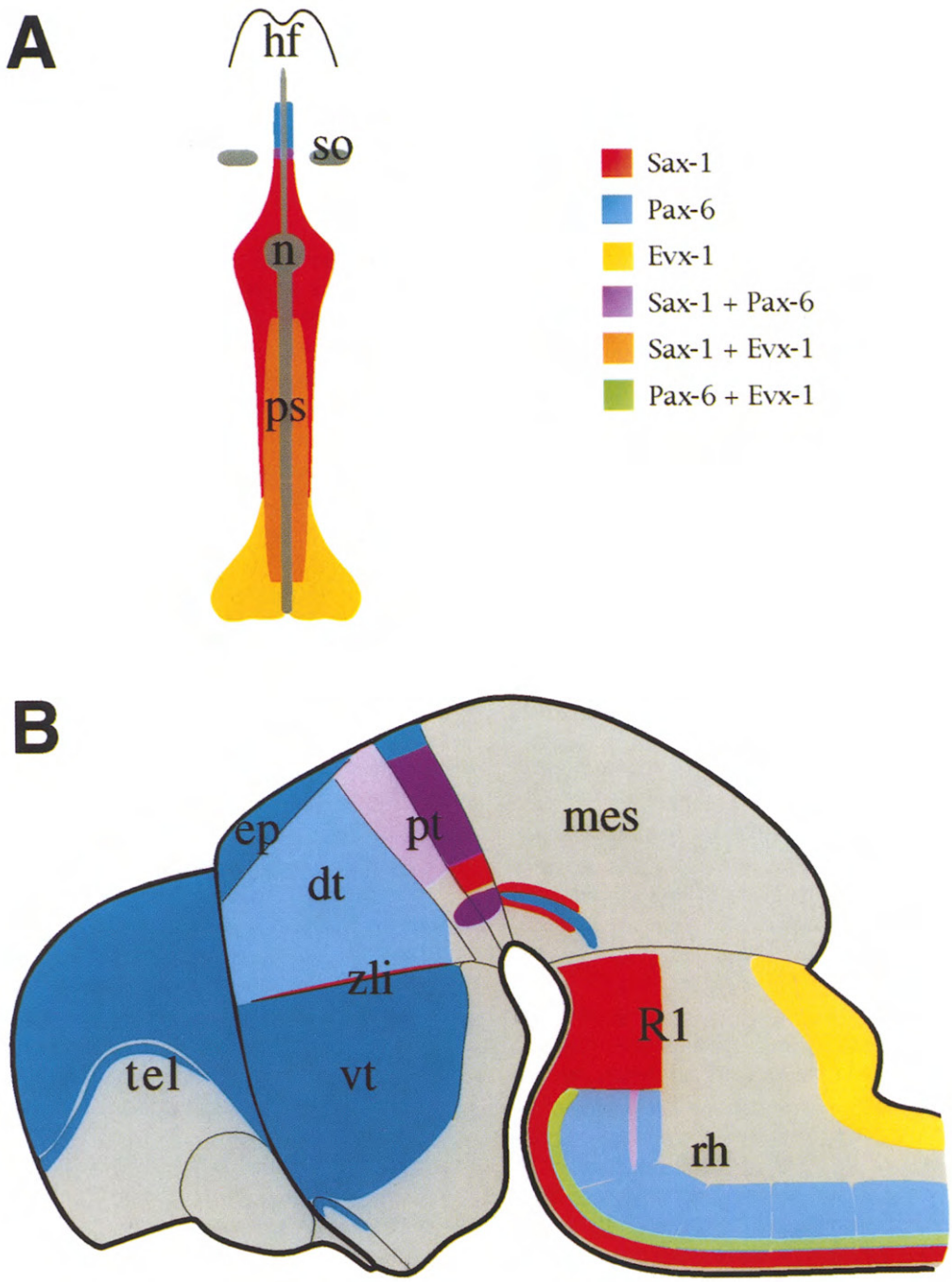

Fig. 9. Schematic representation of *Sax-1*, *Evx-1* and *Pax-6* expression domains. (A) Dorsal view on an idealised amniote embryo at the 1-somite stage showing the successive expression of *Evx-1*, *Sax-1* and *Pax-6* in a posterior-anterior gradient. (B) Lateral view on a day-11 embryonic brain. The craniocaudal and dorsoventral extent of the expression domains is drawn. Please note that differences in mediolateral dimension are not considered. Expression levels are represented by different colour intensity.

tion of these cells into the neural lineage is consistent with its transient expression in the nascent neuroepithelium. Initiated early after the application of retinoic acid, transcript levels decrease again as the differentiation proceeds.

3.2. Differentiation of the CNS

Later in organogenesis, in a second phase *Sax-1* was found to be expressed in several domains in the anterior CNS. Along the spinal cord up to the posterior hindbrain, transcripts were located in pairwise stripes in the

ventrolateral part of the neural tube, the stronger signal residing just adjacent to the ventral horns. *Sax-1*, therefore, like most *Pax*-genes (Gruss and Walther, 1992) and homeobox genes as *Evx-1* (Bastian and Gruss, 1990), marks cells at specific dorsoventral and mediolateral positions within the neural tube. The identity of the *Sax-1* positive cells is unclear. However, the ventral shift and final loss of the signal in *Danforths short tail* homozygotes, synchronous with motor neuron markers (Bovolenta and Dodd, 1991), demonstrates the *Sax-1* expression to be part of the notochord-dependent, ventral differentiation program in the neural tube.

Looking further cranial, in rhombomere 1, the *Sax-1* signal extended, labelling the whole ventral part of the neural tube. *Sax-1*, in this respect, behaves different from *Evx-1* or *Pax-6*, other genes expressed in specific dorsoventral positions in spinal cord and posterior hindbrain (Fig. 9B): the strong ventral *Evx-1* signal, located just dorsal to the ventral *Sax-1* band, and the broad *Pax-6* staining appeared delimited by rhombomere 2 at the stages analysed. While *Sax-1* appears too late to confer positional identity analogous to the *Hox* genes in the caudal rhombomeres, its broad expression in the basal plate of rhombomere 1 suggests an important role within the genetic program underlying the specific differentiation of this anteriormost rhombomere.

Expression of *Sax-1* was also found in localised areas of midbrain and forebrain. Particularly in the ventral midbrain, where *Sax-1* and *Pax-6* were expressed in longitudinal stripes, the expression domains of both genes appear complementary (Fig. 9B): *Pax-6* in a single band ranging approximately through the caudal two thirds of the mesencephalon, and *Sax-1* in two stripes in the cranial two thirds, probably enclosing the *Pax-6* positive cells.

A recently extensively debated hypothesis is the organisation and possible segmentation of the forebrain. Morphological and molecular evidence more and more favours the neuromeric model, suggesting the subdivision of the forebrain into four (Figdor and Stern, 1993) or six transverse segments (Bulfone et al., 1993). The expression domains of *Pax-6* and *Sax-1* in the diencephalon are in good agreement with the proposed boundaries (Fig. 9B). *Pax-6* signals were mainly found in the alar plate: strongly in epithalamus, ventral thalamus and posterior pretectum; weakly in the dorsal thalamus and the anterior pretectum. *Sax-1*, in contrast, was located mainly around axon tracts marking major subdivisions within the embryonic brain: firstly, the posterior commissure at the border to the midbrain; and, secondly, the area of the zona limitans intrathalamica separating dorsal and ventral thalamus, where *Sax-1* may overlap with either *Nkx-2.2* (Price et al., 1992) or *Wnt-3a* (Roelink and Nusse, 1991). In addition, *Sax-1* was expressed in dispersed cells in the anterior pretectum and a patch of cells in the basal plate of the pretectum, regions also positive for *Pax-6*. Both *Pax-6* and *Sax-1*, therefore, respect the boundaries between pretectum and dorsal thalamus and between dorsal thalamus and ventral thalamus, which the supporters of the neuromeric theory agree in describing as neuromere borders.

The expression pattern of the murine homeobox gene *Sax-1* during embryonic development suggests two different functions for the gene product. Initially, it may be involved in the formation of the posterior neuroectoderm, by marking cells competent for differentiation into neuroectoderm, by specifying cells towards the neuroectodermal fate or by conferring posterior identity to neural precursors. Later in development it may specify subsets of neurons destined for a certain function, a role also proposed for the *Drosophila* homologue *NK-1/S59*.

4. Experimental procedures

4.1. Sequence analysis

A 76-bp homeobox fragment of mouse *Sax-1* was amplified from 1 μg genomic mouse DNA isolated from NMRI spleen by polymerase chain reaction (PCR), using moderately degenerated primers (FRS16: ACCMGITAICTITCIGTGTGYGA, FRS17: TCCGGGRTKCTGCTTYTTCCA) based on the *CHox3/cSax-1* sequence (Rangini et al., 1989). Positive clones were identified by hybridisation to a *Sma*I-*Sma*I *cSax-1* homeobox containing probe. The PCR fragment of mouse *Sax-1* was then used to screen an embryonic day 8.5-pc mouse cDNA library cloned into λgt10 (Fahrner et al., 1987). The hybridisation was performed under stringent conditions at 42°C in 1 M NaCl, 1% SDS, 50% formamide. Two positive clones were purified and their inserts cloned into Bluescript vector (Stratagene). Subclones were created following digestion with appropriate restriction endonucleases and sequenced from single or double stranded DNA preparations (T7 kit, Pharmacia).

4.2. Whole mount in situ hybridisation

Digoxygenin-UTP-labelled antisense RNA probes were synthesised by transcription from linearised pBluescript (Stratagene) using the nucleotide mix from Boehringer Mannheim. The probe for mouse *Sax-1* corresponds to the 552-bp *Stu*I-*Eco*RI fragment encompassing the homeobox; for *Pax-6* and *Evx-1*, the probes of the initial expression studies (Bastian and Gruss, 1990; Walther and Gruss, 1991) were used. The whole mount in situ hybridisation followed the protocol in Wilkinson (1992).

Photographs of the early stages and of details were taken on a Zeiss Axiophot microscope, using differential interference contrast. Larger embryos were photographed on a Zeiss Stemi SV11 dissecting microscope at darkfield illumination.

4.3. Sections

After whole mount in situ hybridisation, embryos up to day 9.5 pc were embedded into paraffin following the basic guidelines in Kaufman (1992): the embryos were refixed in 4% paraformaldehyde/PBS (30 min), dehydrated in ethanol (1–3 min each in 70%, 80%, 90%, twice 96%, twice 100% ethanol), transferred into paraplast plus (1–3 min each in 1:1 ethanol/Xylolersatz, 100% Xylolersatz, 1:1 Xylolersatz/paraplast plus 60°C, three times paraplast plus 60°C) and, finally, embedded into paraplast plus under a dissecting microscope; 8 μm sections were taken on a Reichert-Jung 2040 microtome, deparaffinised and mounted in Eukitt.

Older embryos were embedded in gelatine/albumin, fixed by glutaraldehyde, sectioned to 30 μm on a Pelco 101 vibratome and immediately mounted in Moviol (Hoechst). Sections were photographed on a Zeiss Axiophot microscope using differential interference contrast.

4.4. Cell culture

P19 EC cells were cultured in DMEM supplemented with 10% fetal calf serum, 400 mM glucose, 200 mM glutamine and antibiotics. Differentiation into the neural lineage was induced as described by Jones-Villeneuve et al. (1982), growing the cells in bacterial dishes in the presence of 3×10^{-7} M 13-cis retinoic acid for 4 days to allow aggregation, then plating them back into tissue culture dishes in culture medium without retinoic acid. Neuronal processes became frequent after 10 days of differentiation.

4.5. Northern blot

Total cellular RNA from cells was prepared according to Chomczynski and Sacchi (1987). For a Northern blot, 10 μg were applied per lane. Gel electrophoresis was performed in 1× MOPS, the gel supplied by 0.67% formaldehyde. After electrophoresis, the gel was equilibrated in 20× SSC and the RNA transferred to Qiabrene nylon membrane. After drying and UV crosslinking, the filters were prehybridised, hybridised in 0.5 M Sodiumphosphate (NaPi), 7% SDS, 1 mM EDTA at 65°C and washed in 40 mM NaPi, 1% SDS at 65°C, according to Church and Gilbert (1984). The radioactive probe was labelled by random oligo labelling using the random prime kit (Amersham).

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