The spatial and temporal dynamics of \textit{Sax1 (CHox3)} homeobox gene expression in the chick’s spinal cord

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\textbf{SUMMARY}

\textit{Sax1} (previously \textit{CHox3}) is a chicken homeobox gene belonging to the same homeobox gene family as the \textit{Drosophila NK1} and the \textit{honeybee HHO} genes. \textit{Sax1} transcripts are present from stage 2 H\&H until at least 5 days of embryonic development. However, specific localization of \textit{Sax1} transcripts could not be detected by in situ hybridization prior to stage 8, when \textit{Sax1} transcripts are specifically localized in the neural plate, posterior to the hindbrain. From stages 8 to 15 H\&H, \textit{Sax1} continues to be expressed only in the spinal part of the neural plate. The anterior border of \textit{Sax1} expression was found to be always in the transverse plane separating the youngest somite from the yet unsegmented mesodermal plate and to regress with similar dynamics to that of the segregation of the somites from the mesodermal plate. The posterior border of \textit{Sax1} expression coincides with the posterior end of the neural plate. In order to study a possible regulation of \textit{Sax1} expression by its neighboring tissues, several embryonic manipulation experiments were performed. These manipulations included: removal of somites, mesodermal plate or notochord and transplantation of a young ectopic notochord in the vicinity of the neural plate or transplantaion of neural plate sections into the extraembryonic area. The results of these experiments revealed that the induction of the neural plate by the mesoderm has already occurred in full primitive streak embryos, after which \textit{Sax1} is autonomously regulated within the spinal part of the neural plate.

Key words: chicken embryo, homeobox, spinal cord, notochord, expression, \textit{Sax1 (CHox3)}

\textbf{INTRODUCTION}

In the chick, gastrulation via the primitive streak starts at stage 3 H\&H (Hamburger and Hamilton, 1951). The first cells to ingress into the lower layer are the endodermal cells which are followed by the ingress of mesodermal cells that form the middle layer between the epiblast and hypoblast (Bellairs, 1986; Nicolet, 1971; Stern, 1991). At stage 4 H\&H, the primitive streak acquires its full length with Hensen’s node at its anterior end. The mediostral triangle of the node is the anlage of the notochord, and the epiblast rostral to it is the future neural plate (Selleck and Stern, 1991). On both sides of the anlage of the notochord are the cells that after ingressation into the middle layer will form the somites on each side of the ingressed notochord (Schoenwolf, 1979). The somites segregate from the mesodermal plate in an orderly manner, rostral to caudal (Ooi et al., 1986). As development proceeds along the anteroposterior axis, the notochord induces the ventral part of the already formed neural tube to form the floor plate and together with the floor plate, induces motor neurons (Jessel and Dodd, 1992; Ruizi i Altaba and Jessel, 1993), while other, presumably dorsal signals induce the dorsal part of the neural tube (Goulding et al., 1993).

One of the characteristics of the above directional morphogenetic processes is a series of induction interactions between neighboring tissues. A major subject in contemporary research are the molecules that are involved in these inductions, which include secreted molecules and their receptors (reviewed in Jessel and Melton, 1992; Kingsley, 1994; see also Niswander et al., 1993; Riddle et al., 1993; Thaller and Eichele, 1987) and transcription factors that respond to the above factors and transmit the induction signal (reviewed in Gruss and Walther, 1992; McGinnis and Krumlauf, 1992; see also Dolle et al., 1993; Izpisua-Belmonte et al., 1993). A major group of genes which encode transcription factors that are involved in early embryonic development are the homeobox genes, which contain a conserved 183 bp long coding sequence, termed the homeobox (McGinnis and Krumlauf, 1992). Much of the specificity of the homeobox gene products, including their binding to specific DNA sequences, lies within the region
encoded by the homeobox, termed the homeodomain (Scott et al., 1989). Classical and molecular genetic studies of the homeobox genes showed that they function coordinately to initiate and maintain developmental decisions within defined spatial and temporal boundaries of the embryo (reviewed in Kessel and Gruss, 1990; Krumlauf, 1993; McGinnis and Krumlauf, 1992).

Numerous homeobox genes are expressed in the central nervous system (CNS) of vertebrates during its development (Krumlauf, 1993; Wilkinson, 1993). Most of the genes in the four Hox complexes are expressed in the hindbrain and in the spinal cord. The relative position of the genes within the Hox complexes correlates with their anteroposterior pattern of expression in the embryo (reviewed in McGinnis and Krumlauf, 1992). The anterior border of the expression of Hox genes is usually sharp and, in the hindbrain, the expression is colocalized with the borders of rhombomeres (Krumlauf, 1993). It was also shown that even before the boundaries of the rhombomeres become visible, the expression of HoxB-1 and rhombomeres identity are already autonomous in the hindbrain (Guthrie et al., 1992).

The homeobox gene, HbxE (previously CHoxE; Rangini et al., 1991) and genes of the Pax gene family (reviewed in Gruss and Walther, 1992) are transcription factors that are probably involved in the differentiation of the hindbrain and spinal cord along the dorsoventral axis at somewhat later stages of development. In the case of the Pax genes it was shown that they respond to signals that come from the notochord (Goulding et al., 1993).

Several additional chicken homeobox genes that are expressed in the neural tube have been cloned (reviewed in Fainsod and Gruenbaum, 1994). One such gene, which is not included in the chicken Hox complexes is Saxl (previously CHox3; Rangini et al., 1989). Saxl is conserved in evolution, since the Drosophila NK1 (Kim and Nirenberg, 1989) and the honeybee HHO genes (Waldorf et al., 1989) contain a highly homologous homeodomain.

The temporal pattern of expression of Saxl was studied by developmental RNA blot analysis (Rangini et al., 1989). Saxl codes for five transcripts, which are probably the result of alternative splicing, since different genomic probes identify different subsets of transcripts. These Saxl transcripts were detected from the time the egg is laid until 5 days of development. In the present report, we show that Saxl is exclusively expressed in the spinal part of the neural plate at a relatively early stages. Transcripts of Saxl were found to become spatially localized at stage 8 and their anterior limit marks the border between the spinal part of the neural plate and the future hindbrain. At later stages, the anterior border of expression gradually shifts continuously in a caudal direction at the same pace as the segregation of the somites from the mesodermal plate. However, once the spinal part of the neural plate is induced, Saxl transcription is autonomously regulated within it.

**MATERIALS AND METHODS**

**Embryos**

Commercial Yarkon Tint hens’ eggs were incubated at 38°C in a humidified incubator. Embryos were isolated in Ringer’s solution, staged according to Hamburger and Hamilton (1951) and transferred onto fresh vitelline membranes which were isolated from unincubated eggs and stretched around glass rings (New, 1955). Each glass ring encompassing the embryos was put in a small Petri dish containing some solid egg albumen. The various microsurgical manipulations were performed in these dishes after a 1-2 minutes exposure of the embryo to 0.15% trypsin in PBS. After the operation, the medium was aspirated and the dishes were incubated at 38°C for the desired length of time.

**Removal of the newly formed somites**

Embryos of stages 8-10 H&H were placed ventral side up. An endodermal flap, covering the most posterior three somites of one side, was carefully peeled off and folded sidewards and the exposed somites were cut out and discarded. The endodermal flap was then put back into its place and the embryos were incubated at 38°C for additional 6 hours.

**Removal of the mesodermal plate**

Embryos of stages 8-11 H&H were placed ventral side up. The endoderm caudal to the last somite was carefully peeled from the segmental plate on one side and pushed sidewards. A section of the segmental plate either corresponding in length to three already formed posterior somites (approximately 300 µm) or including all the non-segmental tissue until Hensen’s node was isolated and discarded. After the operation, the endoderm was put back on the operated area and the embryos were incubated at 38°C for 5.5-6.75 hours.

**Transplantation of a young notochord into the vicinity of the neural plate**

Pairs of embryos, one of stage 7-9 H&H and the other of stage 9-11 H&H were placed on the same vitelline membrane ventral side up. The younger embryo served as the donor of the notochord, while the older embryo was the recipient. The posterior portion of the notochord from behind the youngest somite down to Hensen’s node, was isolated and transferred to the older embryo. In the recipient embryo, a longitudinal incision was made in the endoderm and then deepened to separate the notochord from the segmental plate. The younger notochord fragment was inserted into the slit, while care was taken to keep its original orientation when transplanted adjacent to the host’s notochord and neural palate. The embryos were incubated at 38°C for additional 5-6 hours.

**Formation of notochord-less embryos.**

Embryos at stage 4 H&H were used. The embryos were placed ventral side up and Hensen’s node was cut out and discarded. The operated embryos were incubated at 38°C for 24 hours. About 20% of the embryos did not regenerate their normal Hensen’s node and were recognized by having only one row of large median somites underneath most or all the neural plate. A similar phenomena was described in spontaneously occurring notochord-less embryos (Stern and Bellairs, 1984). These embryos were taken for further analyses.

**Neural plate isolation**

Embryos of stages 8-12 H&H were placed dorsal side upwards. The right lateral half of the neural plate, caudal to the last somite, was isolated with the aid of a micro-knife (15° angle). The isolated fragment corresponded in length either to three somites (approximately 300 µm) or to the entire section between the last somite and Hensen’s node. The isolated neural plate fragment was transferred to the anterior extraembryonic area, lateral to the forebrain and inserted into the middle layer underneath the ectoderm.

**In situ hybridizations**

In situ hybridizations with 35S-labeled probes were performed exactly as described in Rangini et al. (1991). Whole-mount in situ hybridization was performed according to the method of Wilkinson (1992).
with some modifications. Chick embryos were dissected out in PBS and fixed for 2-20 hours in 4% paraformaldehyde in PBS, washed twice in PBT (PBS; 0.1% Tween 20), dehydrated for 5 minutes each with 25%, 50%, 75% methanol in PBT and twice with 100% methanol. Embryos were then stored at −20°C. Rehydration of the embryos was done by transferring them through the same methanol series in reverse order followed by 2 washes with PBT. Bleaching was performed with 6% hydrogen peroxide in PBT for 1 hour, followed by three washes with PBT. The embryos were digested with 10 μg/ml of Proteinase K in PBT containing 0.05% SDS for 6-15 minutes, depending on the developmental stage of the embryo. After washing once with freshly prepared 2 mg/ml glycine in PBT and twice with PBT, the embryos were rinsed in fresh 0.2% glutaraldehyde and 4% paraformaldehyde in PBT for 20 minutes and washed again with PBT for two more times. Prehybridization was in 1 ml of 50% formamide, 5× SSC pH 4.5, 1% SDS, 50 μg/ml yeast RNA and 50 μg/ml heparin at 70°C for 1 hour. Digoxigenin-labeled RNA probes were transcribed from Sax1 templates (Fig. 1A) in Bluescript vectors using T7 or T3 RNA polymerases. The labeled probes were added to fresh prehybridization buffer at a final concentration of ~1 μg/ml and incubated with the embryos overnight at 70°C. Following hybridization, the embryos were twice washed for 30 minutes at 70°C in 50% formamide, 5× SSC pH 4.5, 1% SDS, once in a 1:1 mixture of the former solution with 0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1% Tween 20 and three times in the later solution. Embryos were then incubated twice, 15 minutes each, with 100 μg/ml of RNase A in 0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1% Tween 20 at 37°C, followed by a wash with the same buffer without the enzyme, then three washes with 50% formamide, 2× SSC pH 4.5, the first at ~22°C and the other two at 65°C, which were followed by three washes with TBST (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH 7.5, 0.1% Tween 20 and 2 mM Levamisole). Blocking of non-specific binding of antibodies was in 10% lamb serum in TBST for 90 minutes. Anti-digoxigenin alkaline phosphatase-conjugated antibodies (Fab') were preabsorbed for 1 hour with powder of chick embryos (precipitated with acetone, 3 mg/μl of 150 units/μl antibody) in TBST containing 1% lamb serum. Embryos were then incubated overnight in 1:2,000 dilution of the antibody in TBST containing 1% lamb serum at 4°C. After three washes, 5 minutes each, and five washes, 10 minutes each, with TBST they were washed three times for 10 minutes with NTMT (0.1 M NaCl, 0.1 M Tris-HCl pH 9.5, 50 mM MgCl₂, 0.1% Tween 20, 2 mM Levamisole). The solution was then changed into NTMT containing 0.33 mg/ml nitro-blue tetrazolium salt (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and the embryo was then incubated for 90-120 minutes in the dark. The reaction was monitored microscopically and stopped by two washes with PBS, fixation in 4% paraformaldehyde for 1 hour, three more washes in PBS, one wash in PBS containing 50% glycerol and one with PBS containing 80% glycerol. The embryos were mounted in 80% glycerol in PBT and photographed using a Zeiss Stemi SV11 stereo microscope and a contax 167 MT camera.

Some of the embryos were then washed three times, 10 minutes each, with PBS, embedded in 7% gelatin and fixed overnight in 4% paraformaldehyde in PBS. The embryos were then sectioned, using a vibratome (series 1,000). The 70 μm-thick sections were placed on gelatin-coated slides, mounted with entellan and photographed using a Zeiss Axiovert 135TV microscope.

RESULTS

Sax1 expression during the first days of chick embryogenesis

Developmental northern analyses revealed that Sax1 transcripts are already present at stage 2 H&H of embryonic development (Rangini et al., 1989). In order to localize Sax1 transcripts spatially during the stages of gastrulation and early neuralization, in situ hybridization analysis was performed on whole-mounted as well as on serially sectioned embryos of stages 4-7 H&H, using Sax1 probes A or B (Fig. 1A). The results of these experiments revealed that at the above stages there was no localized signal of hybridization (data not shown).

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(A) Partial restriction map of the Sax1 genomic clone that was used in this study. The homeobox region is shown as a rectangle. The arrow above the homeobox shows the direction of the transcription. The locations of probes A and B are marked above the restriction map. Abbreviations B, BamHI; E, EcoRI; K, KpnI; S, Smal. (B) Whole-mount in situ hybridization of Sax1 to stage 10 H&H chick embryo, using a digoxigenin-labeled Sax1 probe B. The hybridization signal appeared in the neural plate as a dark blue stain of the alkaline phosphatase reaction. hn, Hensen’s node; ps, primitive streak; n, notochord; np, neural plate; s, somites; st, spinal tube; ys, youngest somite. Bar represents 500 μm.
approximately at the level of the 4th somite, which marks the border between the future brain and the spinal cord (Fig. 2A). Since that stage onwards, the anterior border of Sax1 transcripts in the neural plate continuously regressed in a posterior direction in a spatial and temporal correlation with the segregation of somites from the mesodermal plate, and was always at the same plane as the border line between the youngest somite and the mesodermal plate (Figs 1B, 2). The posterior border of Sax1 transcripts continuously shifted backwards with the posterior elongation of the neural plate and always coincided with the posterior end of the neural plate. No hybridization signal was observed when the in situ hybridization was performed on control embryos using the sense strand of either probes A or B (data not shown).

In order to verify the spatial expression of Sax1, in situ hybridization was performed on frontal sections of stages 10-11 H&H embryos, using a [35S]-labeled probe (Fig. 3A, B). The results of these experiments revealed, again, that the anterior border of Sax1 expression is roughly in the same transverse plane as the posterior border of the youngest somite. A control probe of 35S-labeled Sax1-sense RNA did not show hybridization signal to neighboring sections (data not shown).

The in situ hybridization to both whole-mounted embryos (Figs 1B, 2) and frontal sections (Fig. 3A, B) also revealed that there is probably a quantitative posteroanterior gradient of the amounts of Sax1 transcripts in the neural system. The strongest signal was observed posteriorly while its intensity declined toward the anterior border of expression. The spatial localiza-
tion of the various *Sax1* transcripts seems to be similar, since probe B (Fig. 1A), which lacks the homeobox and recognizes the 1.9 kb transcript, and probe A (Fig. 1A), which contains the homeobox and recognizes all five *Sax1* transcripts (Rangini et al., 1989), both showed a similar spatial pattern of hybridization. However, in most experiments, the intensity of the in situ hybridization signal was higher when probe A was used.

*Sax1* expression along the dorsoventral axis

In order to study the pattern of *Sax1* expression along the dorsoventral axis of the embryo, the whole-mounted hybridized embryos were serially cross-sectioned at 70 µm. Examination of these sections revealed that the intensity of hybridization of the probes to *Sax1* transcripts was roughly uniform in the neural cells along the dorsoventral axis. No hybridization signal was detected in the neural folds, which are to form the future neural crest (Fig. 4). In some cases, the signal seemed to be stronger either at the dorsolateral or the ventral section of the neural tube, but it might have been a result of oblique sectioning of the embryos, with a weaker expression representing a slightly more anterior part of the tube. To support these observations further, stage 12 H&H embryos were first serially sectioned and then in situ hybridized with a 35S-labeled RNA probe. The results of these experiments again demonstrated that *Sax1* transcripts were restricted to the neural plate cells with a more or less uniform hybridization signal along the dorsoventral axis, which was restricted from the neural crest region (Fig. 3C,D). Similar experiments with stage 8 H&H or stage 15 H&H embryos gave identical results (data not shown). Control probes A or B of 35S-labeled *Sax1*-sense RNA did not give a signal when hybridized to neighboring sections (data not shown).

*Sax1* expression in manipulated embryos

*Sax1* pattern of expression in the neural plate correlates very nicely with the dynamics of the segregation of somites from the mesodermal plates. We therefore decided to check experimentally a possible correlation between the differentiation processes in the neural system and its neighboring mesodermal structures, such as the posterior somites, the non-segmented mesodermal plate and the notochord. We have therefore changed the spatial relations between the neural tube and each of the above structures and checked if and how this would affect *Sax1* pattern of expression.

Somite segregation does not regulate *Sax1* expression

In the first set of experiments (n=5), we wanted to determine whether the unilateral removal of somites will cause the neighboring region of the neural plate to maintain the expression the *Sax1* gene. Three newly formed somites were unilaterally removed from the vicinity of the neural plate and the embryos were further incubated for additional 3 to 6 hours, during which 2-3 new more posterior somites were added on both sides of the neural tube (Fig. 5A). The results of these experiments revealed that *Sax1* expression remained equal on both the operated and the control non-operated sides of the nervous system (Fig. 5B). Thus, the removal of somites did not affect the disappearance of *Sax1* expression in the operated side.
In a second set of experiments (n=10), we tried to avoid any possible contact between the neural plate and defined somites. Thus, we removed unilaterally sections of the mesodermal plate posterior to the already formed somites corresponding to the neural plate at the transition area between the last somite and the mesodermal plate. The embryos were then incubated for about 6 hours and processed for whole-mount in situ hybridization, using a Sax1 probe. The results of the in situ analysis revealed that the spatial distribution of Sax1 transcripts was identical on both sides of the neural tube. Thus, we conclude that the time of the operation Sax1 pattern of expression was already determined and the interaction with a young notochord did not affect it.

In the above experiments with the implanted young notochord, the neural system continued to be under the influence of the original notochord or its precursor cells. Therefore, we decided to test whether the picture would be altered by avoiding any contact between the induced neural tissue and the notochord during its entire differentiation.

In a complementary set of manipulations (n=3), the region of the Hensen’s node, from which the notochord develops was removed from stage 4 H&H embryos (Fig. 7A), in which the induction of the neural tissue is known to already have taken place (Selleck and Stern, 1991, 1993). The embryos were further incubated until they reached stages 9-11 H&H. Notochord-less embryos were formed which contained only a single row of median somites underneath the neural plate (Fig. 7B). The pattern of Sax1 expression was found not to be influenced by the total lack of notochord (Fig. 7C-F). Thus we conclude that neither the absence nor the presence of a notochord affect Sax1 pattern of expression.

Autonomous regulation of Sax1 expression within the neural plate

All the above experiments point to the possibility that Sax1 expression is autonomously regulated within the spinal section of the neural plate. To verify this possibility, longitudinal stripes of one half of the neural plate from the region that expresses Sax1 were cut out from embryos at stages 8-12 H&H and transplanted into the anterior extraembryonic region, far from conventional neighboring structures, while the other half of the neural plate was left in place (Fig. 8A,D). Some of the neural plate corresponded in length to future three somites while others included the entire length of the neural plate. In some of the above experiments the transplant was inserted into the extraembryonic region in an anteroposterior orientation conforming with the original orientation of the embryo (Fig. 8A), while in others it was inserted in the reverse orientation (Fig. 8D). After incubation periods of 2.5-6 hours, the spatial distribution of Sax1 transcripts in the transplant was compared to that of the identical intact part of the neural plate.

In all our experiments (n=12), both kinds of transplants proved to have the same pattern of Sax1 expression as the non-manipulated side of the neural plate (Fig. 8B-C, E-F). After a longer incubation period of 16-20 hours, the Sax1 signal disappeared altogether from the transplants as well as from both the corresponding non-manipulated region (data not shown).
DISCUSSION

Most vertebrate homeobox genes are expressed in more than one germ layer (reviewed in Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992; Krumlauf, 1993; Fainsod and Gruenbaum, 1994). Sax1 is unique among those genes since it is currently the only known homeobox gene which during stages 8–15 H&H is exclusively expressed in a defined ectodermal section — the spinal part of the neural plate. Another gene that is specifically expressed in the neural tube is HbxE (Rangini et al., 1991). However, unlike Sax1, HbxE is expressed in the intermediate plate of the neural tube at later stages.

Fig. 5. The expression pattern of Sax1 is not affected by the removal of posterior somites or sections of the mesodermal plate. (A) A schematic view of the operation in which the most posterior three somites were removed from one side of the neural plate. (B) Following 6 hours incubation the embryo was in situ hybridized with a Sax1 probe B. (C) A schematic view of the operation in which either a small piece of the mesodermal plate or most of the mesodermal plate were removed from one side of the neural plate. Following 6 hours incubation the embryos were in situ hybridized with a Sax1 probe B. The results of the hybridization of the operated side in the embryos in which a small piece (D) or a large piece (E) of the mesodermal plate were removed were similar to those of the non-operated side. The arrows in B point to the limits of the region from which the somites where removed. The arrows in D and E point to the region from which the mesodermal plate was removed. Bar represents 500 μm.
stages of development and is probably involved in differentiation events along the dorsoventral axis of the neural tube.

At stage 8− Sax1 is first localized and its anterior border is behind the 4th visible somite. The neural plate section above the 4th somite, which will become a part of the hindbrain (Jacobson, 1992), does not express Sax1. The timing, as well as the pattern of expression of Sax1 in the spinal region, resembles those of Hox B5, which is expressed in mice at a similar developmental stage (3−4 somites), starting at the border between head and trunk and spreading backwards (Wilkinson et al., 1989). Wilkinson et al. (1989) suggested that this limit of expression may correlate with a transition in CNS organization, which may apply also to the limits of expression of Sax1. However, in contrast to Hox B5, Sax1 is the only known gene with an anterior border that continuously regresses in a posterior direction and roughly coincides with the plane where the youngest somite has just segregated from the mesodermal plate (Figs 1, 2). The posterior border of Sax1 transcripts always coincides with the posterior border of the neural plate merging into the primitive streak.

Developmental northern analysis has shown that Sax1 is already expressed in blastoderms at the early primitive streak and head fold formation stages, namely stages 2−5 H&H (Rangini et al., 1989). However, Sax1 transcripts could be spatially localized by in situ hybridization from stage 8− H&H and on, after the first 3−4 pairs of somites have segregated from the mesodermal plate. Since the in situ hybridization technique gives a clear signal only when enough transcript(s) are localized in a spatially restricted manner, it is likely that prior to stage 8− H&H either most or all cells in the blastoderm express low amounts of Sax1 or that the levels of the transcripts in specific cells are below the limits of the sensitivity of the technique.

Looking for a connection between Sax1 expression and its contemporary morphogenetic events, we sought a correlation with the following events: the closure of the neural plate into a tube, the segregation of the somites from the mesodermal plate, and the process of formation and maturation of the notochord. The experiments in this study were planned to address each of the mentioned possibilities except for the first one, as it became clear that even in normal embryos a signal could be detected in the closed section of the neural tube in stages 14−15 H&H, while in embryos of stages 8−9 H&H, a section of an open neural plate was found to have lost its signal (data not shown).

The timing and the mechanism of the induction of the posterior neural plate in avian embryos are not yet entirely clear. Storey et al. (1992) have suggested two alternative mechanisms by which the more posterior regions of the CNS could be generated: (i) homeogenetic induction by the anterior sections of the neural plate which have already been induced, spreading posteriorly in the epiblast, and (ii) elongation of posterior neural primordia that have been induced at a very early stage, and which are probably confined into a relatively small ectodermal area anterior and lateral to Hensen’s node (Schoenwolf, 1991). Whatever the mechanism might be, it is clear that from stage 5 H&H and on, the formation of the spinal part of the neural plate, as demonstrated by Sax1 expression, does not require the presence of either normal Hensen’s node or notochord. This is why in either case the removal of the node, which results in partially notochord-less embryos, does not interfere either with the formation of the spinal section of

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**Fig. 6.** The expression pattern of Sax1 is not affected by the presence of an ectopic young notochord near the neural plate. The notochord was dissected from a stage 8 H&H embryo and transplanted to the vicinity of the neural plate of a stage 9 H&H embryo (A, B). Following 6 hours incubation the embryo was in situ hybridized with a Sax1 probe B (C). The arrows in C point to the limits of the region where the ectopic notochord was transplanted. Bar represents 500 µm.
Fig. 7. The expression pattern of *Sax1* is not affected in notochord-less embryos. The Hensen’s node region of a stage 4+ H&H chick embryo was removed (A). The operated embryos were incubated at 38°C for 24 hours and in situ hybridized with a *Sax1* probe B (B). The embryo shown in B was serially cross sectioned at 70 µm. The position of the cross sections shown in C-F is marked in B. (C) Section from the region containing single medial somites shows no expression of *Sax1*. (D) Section from the region of the neural plate with a mesodermal plate. (E) Section from the region of the primitive streak where Hensen’s node should have been located. Note the absence of a notochord in all these sections. The numbers in A represent: (1) head process, (2) primitive knot, (3) primitive groove, (4) primitive ridge. Abbreviations: PS, primitive streak; NT, neural tube; NC, neural crest; NP, neural plate; MS, medial somite. Bar represents 500 µm in B and 50 µM in C-F.
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Fig. 8. The pace of regression of the anterior border of Sax1 is not affected when sections of the neural plate that express Sax1 are transplanted to an extraembryonic area. A stripe corresponding to the length of most of the neural plate was dissected from one side of the neural plate and transplanted to an extraembryonic tissue near the head, either in its original anteroposterior orientation (A) or in the reverse orientation (D). Following 3 (B,C) or 6 (E,F) hours incubation, the embryos were in situ hybridized with a Sax1 probe B (B,C and E,F). Panels B,C and E,F show the same embryos, but taken with different background colors to emphasize the position of the hybridization signal with respect to the morphology. Upper arrow points to the transplant. Lower arrow points to the region from which the transplant section was taken. Bar represents 500 µm.

the neural plate, or with its extension above the single row of medial somites, all the way down to the defective streak. In other words, the expression of Sax1 seems to be already determined simultaneously with the induction of the spinal region of the neural plate, after which it is not affected by the notochord. The experiments in which the neural plate was removed from the influence of either notochord or somites as well as those in which a young notochord was implanted near the signal transition area, all point to the same conclusion. The gradual disappearance of the hybridization signal from the anterior towards the posterior end of the spinal neural plate, probably exhibits the temporal gradient of differentiation which is initially reached in the anterior section of the developing spinal cord and gradually shifts posteriorly.
Sax1 seems to be more or less expressed in all the cells of a given transverse plane of the neural plate but not in the neural folds, which are the primordia of the neural crest. In contrast, other genes that encode for transcription factors such as HoxE and the Pax genes, which are also expressed in the spinal cord of early embryos, have a restricted dorsoventral pattern of expression. Experimental evidence points to the impact that the notochord has on the expression of the Pax genes (Goulding et al., 1993). Thus, the remarkable differences between Sax1 and Pax genes is that the expression of Sax1 in the spinal cord is determined earlier, is not limited to a certain section of the spinal cord and is probably related to an initial induction or determination of the spinal part of the neural plate. This state of determination, as signaled by Sax1 expression, spreads from the anterior end of the spinal neural plate posteriorly and is probably related to the physiological age of the tissue. In contrast, the expression of the Pax genes, which is restricted along the dorsoventral axis, is somewhat later in development and is related to differentiation processes along the dorsoventral axis within the previously determined spinal cord.

Another prominent morphogenetic change in the embryo that occurs at the period studied is the gradual anteroposterior formation of somites. The dynamics of Sax1 expression follows that of somite formation. Since induction signals between neighboring tissues are common during early embryogenesis, we looked for a possible connection between the neural plate and the forming somites in the form of a signal that might coordinate the differentiation rhythms of the two. The results of our experiments, which included the removal of posterior somites, removal of mesodermal plate or transplantation of sections of the neural plate into the extraembryonic area, where it had neither contact with the somites nor with the mesodermal plate, showed that Sax1 pattern of expression is independent of the above tissues.

Although we can not rule out the possibility of just a coincidence, the results presented here suggest that in the avian embryo there is a developmental clock, yet unknown, which normally determines and coordinates the level of differentiation of all the tissues in a given transverse plane at a given time.

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REFERENCES


Scott, M. P., Tamkun, J. W. and Hartzell, G. W. III (1989). Histological and ultrastructural observations of tail bud formation in the chick blastoderm. Since induction signals in the form of a signal that might coordinate the differentiation rhythms of the two results presented here suggest that in the avian embryo there is a developmental clock, yet unknown, which normally determines and coordinates the level of differentiation of all the tissues in a given transverse plane at a given time.
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