INTRODUCTION

Numerous families of genes encoding transcription factors display early regionalized expression in the developing spinal cord (for review see Tanabe and Jessell, 1996). Of these, certain members of the Pax gene family (paired-type homeobox), particularly those coding for proteins harbouring both a paired domain and a paired-type homeodomain, such as Pax6, exhibit patterns of expression suggestive of their playing a major role in the commitment of neural progenitor cells (for review see Pituello, 1997). In the chicken spinal cord, Pax6 transcripts are first detected in 4-somite-stage embryos (Goulding et al., 1993). From this stage onwards Pax6 is progressively upregulated in the neural tube neighbouring each newly formed somite, but is progressively upregulated in the neuroepithelium neighbouring each newly formed somite.

In the present study, we accumulate data suggesting that this initial activation of Pax6 is controlled via the paraxial mesoderm in correlation with somitogenesis. First, we observed that high levels of Pax6 expression occur independently of the presence of SHH-expressing cells when neural plates are maintained in culture in the presence of paraxial mesoderm. Second, grafting a somite caudally under a neural plate that has not yet expressed the gene induces a premature activation of Pax6. Furthermore, after the graft of a somite, a period of incubation corresponding to the individualization of a new somite in the host embryo produces an appreciable activation of Pax6. Conversely, Pax6 expression is delayed under conditions where somitogenesis is retarded, i.e., when the rostral part of the presomitic mesoderm is replaced by the same tissue isolated more caudally. Finally, Pax6 transcripts disappear from the neural tube when a somite is replaced by presomitic mesoderm, suggesting that the somite is also involved in the maintenance of Pax6 expression in the developing spinal cord. All together these observations lead to the proposal that Pax6 activation is triggered by the paraxial mesoderm in phase with somitogenesis in the cervical spinal cord.

Key words: Pax6, Cervical spinal cord, Paraxial mesoderm, Somitogenesis, Chick embryo

SUMMARY

Pax6 is a paired-type homeobox gene expressed in discrete regions of the central nervous system. In the spinal cord of 7- to 10-somite-stage chicken embryos, Pax6 is not detected within the caudal neural plate, but is progressively upregulated in the neuroepithelium neighbouring each newly formed somite.

In the present study, we accumulate data suggesting that this initial activation of Pax6 is controlled via the paraxial mesoderm in correlation with somitogenesis. First, we observed that high levels of Pax6 expression occur independently of the presence of SHH-expressing cells when neural plates are maintained in culture in the presence of paraxial mesoderm. Second, grafting a somite caudally under a neural plate that has not yet expressed the gene induces a premature activation of Pax6. Furthermore, after the graft of a somite, a period of incubation corresponding to the individualization of a new somite in the host embryo produces an appreciable activation of Pax6. Conversely, Pax6 expression is delayed under conditions where somitogenesis is retarded, i.e., when the rostral part of the presomitic mesoderm is replaced by the same tissue isolated more caudally. Finally, Pax6 transcripts disappear from the neural tube when a somite is replaced by presomitic mesoderm, suggesting that the somite is also involved in the maintenance of Pax6 expression in the developing spinal cord. All together these observations lead to the proposal that Pax6 activation is triggered by the paraxial mesoderm in phase with somitogenesis in the cervical spinal cord.

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INTRODUCTION

Numerous families of genes encoding transcription factors display early regionalized expression in the developing spinal cord (for review see Tanabe and Jessell, 1996). Of these, certain members of the Pax gene family (paired-type homeobox), particularly those coding for proteins harbouring both a paired domain and a paired-type homeodomain, such as Pax6, exhibit patterns of expression suggestive of their playing a major role in the commitment of neural progenitor cells (for review see Pituello, 1997).

In the chicken spinal cord, Pax6 transcripts are first detected in 4-somite-stage embryos (Goulding et al., 1993). From this stage onwards Pax6 is progressively upregulated in the neural tube neighbouring each newly formed somite, whereas it remains absent from the caudal open neural plate (Goulding et al., 1993; Li et al., 1994). After neural tube closure, Pax6 transcripts are present in the entire lateral walls of the mitotically active neuroepithelium, excluding only the ventral midline, where floor plate differentiation is taking place (Goulding et al., 1993; Li et al., 1994; Ericson et al., 1997). The gene then acquires its definitive domain of expression in response to the opposing actions of the signaling molecules controlling dorsoventral patterning of the spinal cord (for review see Tanabe and Jessell, 1996; Pituello, 1997). In the ventral half of the neural tube (basal plate), Pax6 is rapidly down-regulated in cells adjacent to the floor plate (Goulding et al., 1993; Ericson et al., 1997) and subsequently displays a ventrallow-dorsalhigh gradient of expression (Ericson and al., 1997). This specific pattern of expression seems to be tightly controlled by the ventralizing (SHH-mediated) signal from the notochord and floor plate. Thus, grafting a supernumerary notochord or a floor plate completely abolishes Pax6 expression in the adjacent neural tube, while the absence of ventralizing structures is associated with the presence of Pax6 transcripts in the entire ventral neural tube including the ventral midline (Goulding et al., 1993; Ang and Rossant, 1994; Chiang et al., 1996). Likewise, treating neural plate explants with relatively high doses of SHH abolishes Pax6 expression in neural progenitor cells, while progressively lower doses lead to the generation of progenitor subpopulations expressing gradually higher Pax6 levels (Ericson et al., 1997). These data have led to the proposal that graded SHH signaling may generate the gradient of ventrallow-dorsalhigh Pax6 expression observed in the basal plate (Ericson et al., 1997). In the dorsal half of the neural tube (alar plate), Pax6 transcripts are also

Activation of Pax6 depends on somitogenesis in the chick embryo cervical spinal cord

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progressively down-regulated in the vicinity of the roof plate (Goulding et al., 1993; Ericson et al., 1997). The observation that, in vitro, Pax6 can be down-regulated in a dose-dependent manner by activin A (Pituello et al., 1995), together with the presence of activins in the dorsal neural tube (Connolly et al., 1995; Liem et al., 1997), suggests that this dorsalizing factor may participate in the regulation of this gene in the alar plate. Finally, Pax6 expression is switched off in neuroepithelial progenitors as they leave the ventricular zone, except for a small population of cells in the mouse spinal cord that emigrate from the ventral ventricular zone to become located on either side of the floor plate (Walter and Gruss, 1991; Tremblay et al., 1996).

Interestingly, it has been observed that the intensity of Pax6 expression by progenitor cells is predictive of the fate of their neuronal progeny (Ericson et al., 1997). This observation has led to the proposal that, in the ventral spinal cord, the specification of neuronal subtypes occurs via a Pax6-dependent generation of distinct progenitor subpopulations (Ericson et al., 1997). The importance of Pax6 in controlling the identity of ventral progenitors, and thus the identity of ventral neurons, has been emphasized by the analysis of Pax6-deficient mice (Small-Eye (SeY) mutants). In SeY embryos, two classes of ventral interneurons, V1 and V2, originating in the Pax6 expression domain, are affected: no V1 interneurons are generated in the spinal cord of these mutants and the differentiation of the V2 population is impaired (Ericson et al., 1997). The number and/or subtypes of motor neurons is also affected in SeY/SeY embryos, with phenotypic variations depending on the rostrocaudal level considered (Ericson et al., 1997; Osumi et al., 1997). Taken together, these observations emphasize the importance of an accurate regulation of Pax6 expression in the developing spinal cord.

While the findings reported above have substantially advanced our understanding of how the region-specific expression domain of Pax6 in the developing neural tube is set up, the mechanisms by which the gene is initially activated are unknown. We first address this question for a “naïve” part of the neural plate, the post-nodal superficial layer of the sinus venosus (Ericson et al., 1997). The number and/or subtypes of motor neurons is also affected in SeY/SeY embryos, with phenotypic variations depending on the rostrocaudal level considered (Ericson et al., 1997; Osumi et al., 1997). Taken together, these observations emphasize the importance of an accurate regulation of Pax6 expression in the developing spinal cord.

### MATERIALS AND METHODS

**Explant cultures**

Chick eggs, obtained from a commercial source, were incubated for 36 hours in a humidified atmosphere at 38°C. Dissections were performed on embryos at the 7- to 10-somite stage (HH stage 9-10; Hamburger and Hamilton, 1951). The different regions of the neural plate (cf. Fig. 1) were accurately excised using insect needles while mounted on glass in the presence of 1X pancreatin (Gibco) dissolved in Tyrode’s solution (Gibco). The explanted tissues were then cultured in three-dimensional collagen gels (Pituello et al., 1995) or on collagen gel-coated plastic coverslips (Poncet et al., 1996) in medium 199, as previously described (Pituello et al., 1995). Prior to fixation, the cultures were treated with collagenase (Pituello et al., 1995).

**Grafting of somites and presomitic mesoderm**

**Somites**

Host and donor embryos at the 7- to 10-somite stage were pinned dorsal side down, through the area opaca, on Sylgard-coated dishes containing Tyrode’s solution. The host embryos were operated on from the ventral side to preserve the normal relationship of the neural plate with the non-neural ectoderm and the notochord. The last formed somite of 7- to 10-somite stage donors or more anterior somites, according to the indications mentioned in the text, were excised in the presence of 1X pancreatin (cf. Fig. 4A). A piece of the presomitic mesoderm the size of a single somite was cut from the host embryo at around the prospective somite position –III to –IV with or without enzymatic treatment. This level was defined by measuring the size of an already formed somite using a graticule. The unsegmented mesoderm was cut off at a distance corresponding to two or three somites from the last formed somite over a length corresponding to that of one somite. This piece of unsegmented mesoderm was replaced by the somite excised from the donor embryo, and the host embryo was reincubated in the dish at 38°C in a humidified atmosphere for 2.5 hours to 5 hours. The observation that, at the end of the incubation period, the number of somites of the host embryo had increased and, in the case of the oldest ones, the heart was beating, was used as indicators that the grafted embryos had developed correctly. In some cases a roughly somite-sized piece of membrane (type GS, 0.22 μm, Millipore) was grafted on the contralateral side of the host embryo after removal of the paraxial mesoderm as described above.

**Caudal presomitic mesoderm**

A piece of presomitic mesoderm was cut from a donor embryo at the prospective –IV to –V somite level (cf. Fig. 6A). This mesoderm was grafted in different positions, between prospective somite –II and somite +II (cf. Fig. 6A) in a host embryo of 7-10 somites. In control experiments, a Millipore membrane was grafted in the same conditions. The grafted embryos were then reincubated for about 3 hours.

**Pretreatment with anti-SHH-N blocking antibody**

Prior to the graft, the somites were incubated in Tyrode’s solution containing the blocking antibody 5E1, obtained from the Developmental studies Hybridoma Bank. The optimum conditions for antibody fixation were determined by measuring the fluorescence obtained after incubation of notochord explants in SHH-blocking antibodies. Hence notochordal explants were incubated in different dilution of 5E1 supernatant or in protein-A-purified 5E1 IgG (up to 7.5 μg/ml, i.e., a dose twice that used to inhibit SHH-N induction of Pax1 (McMahon et al., 1998) and three times that used to block motor neuron differentiation (Ericson et al., 1996)). After incubation for different periods of time in the blocking antibody, the explants were washed twice and a rhodamine-conjugated anti-mouse IgG was applied for 30 minutes at room temperature. The explants were washed again and the fluorescence was examined. We thus determined that a 30-minute incubation at 37°C in a 1/8 dilution of 5E1 supernatant gave an optimum level of antibody fixation on living notochord cells. We therefore treated the somites under the same conditions and, despite the absence of detectable immunostaining, even using confocal analysis and quantification of fluorescence, grafted them under the neural plate of an embryo at the 9-10-somite stage.

After the appropriate incubation time, the grafted embryos were fixed and in situ hybridization was performed.
Whole-mount antibody staining
The presence of notochordal cells was visualized as previously described (Pituello et al., 1995) using the monoclonal antibody Not1 (Yamada et al., 1991) obtained from the Developmental Studies Hybridoma Bank.

Whole-mount in situ hybridization
Pax6 transcripts were analyzed using the antisense RNA probes prepared as described by Pituello et al. (1995). Chick HNF3β cDNA was provided by A. Ruiz i Altaba (Ruiz i Altaba et al., 1995), chick Shh cDNA was a gift from C. Riddle (Riddle et al., 1993) and chick paraxis cDNA was a gift from E. Olson (Garcia-Martinez et al., 1997). The whole-mount in situ procedure was performed according to the method of Wilkinson (1992).

RESULTS

Neural plate isolated caudally to Hensen’s node develop strong Pax6 expression in vitro
The first question we attempted to answer was whether prospective neuroepithelial cells located behind Hensen’s node in 7-10-somite stage chick embryos are specified to express Pax6. Accordingly, we explanted region C (C, caudal to Hensen’s node) (Fig. 1A-C), cultured it for 24 hours, and looked for Pax6 expression in the explants by in situ hybridisation. As a positive control, we cultured region R (R, rostral to Hensen’s node) isolated from the same embryos (Fig. 1E). Note that Pax6 transcripts were analyzed using the antisense RNA probes prepared as described by Pituello et al. (1995). Chick HNF3β cDNA was provided by A. Ruiz i Altaba (Ruiz i Altaba et al., 1995), chick Shh cDNA was a gift from C. Riddle (Riddle et al., 1993) and chick paraxis cDNA was a gift from E. Olson (Garcia-Martinez et al., 1997). The whole-mount in situ procedure was performed according to the method of Wilkinson (1992).

Neural plate isolated caudally to Hensen’s node transcribes Pax6 at high levels in the absence of ventralizing cells
The development of a high level of Pax6 transcription in region C excised behind the caudal limit of the node prompted us to determine whether this expression was totally independent of the presence of Hensen’s node cells and particularly of derivatives such as notochord and floor plate, both of which are sources of ventralizing signals. To identify Hensen’s node, notochord or floor plate cells in our cultures, we used several markers, including HNF3β transcripts present in Hensen’s node, notochord and floor plate (Ruiz i Altaba et al., 1995), Shh transcripts expressed in notochord and floor plate (Riddle et al., 1993) and the antigen recognized by Not1 antibodies only expressed in notochordal cells (Yamada et al., 1991). Region R, once again used as a positive control, differentiated a floor plate and thus expressed HNF3β (Fig. 2A) and Shh (Fig. 2B). The presence of these SHH-expressing cells may account for the high level of Pax6 transcripts observed in region R (Pituello et al., 1995). In contrast, cultures of region C did not show HNF3β expression. Interestingly, Pax6 displayed a regionalized domain of expression, restricted to one half of the explant (Fig. 1F). There were no obvious differences in the level of transcripts between regions R and C (Fig. 1E and F) but the domains of expression were different; we observed two bands in region R, one on each side of the differentiating floor plate (Fig. 1E; Pituello et al., 1995), whereas in region C only a single continuous band of positive cells could be seen. The presence of Pax6 transcripts in region C isolated in vitro indicates that the neural plate may be specified very early to express Pax6.

Neural plate isolated caudally to Hensen’s node expresses Pax6 high levels in the absence of ventralizing cells
(Fig. 2C), Shh (Fig. 2D) or Not1 staining (data not shown) and therefore did not contain Hensen’s node cells or derivatives. Although this observation does not allow the conclusion that Pax6 expression is independent of Hensen’s node, since the latter may have instructed the cells earlier (see Discussion), it is nevertheless clear that high levels of Pax6 transcription can be obtained in the absence of ventralizing cells.

Pax6 expression is up-regulated in vitro by the paraxial mesoderm
Two non-exclusive hypotheses may be proposed to explain why high levels of Pax6 expression can be observed in vitro in the absence of ventralizing signals while in vivo, under the same conditions, Pax6 is absent or only weakly expressed (Goulding et al., 1993; Ang and Rossant, 1994; Chiang et al., 1996): Pax6 may continue to be repressed in vivo or our cultures may contain a population of non neural cells that are responsible for Pax6 up-regulation. Particularly relevant to this hypothesis is the fact that fate map analysis reveals that the prospective paraxial mesoderm behind Hensen’s node underlies, and is not completely separated from, the caudally located neural plate (Catala et al., 1996). To check for the presence of precursors of the paraxial mesoderm in our cultures, we used paraxis, a marker of the paraxial mesoderm and newly formed somite (Burgess et al., 1995; Garcia-Martinez et al., 1997). As clearly shown in Fig. 3A, each explant contained a small group of cells expressing paraxis, demonstrating the presence of paraxial mesoderm in neural plate cultures isolated caudally to the node.

The next issue was thus to determine whether the paraxial mesoderm was able to upregulate Pax6 expression in the neural plate. To answer this question we performed the following experiment: we dissected both presumptive dorsal halves of the neural plate at the level of Hensen’s node (Fig. 3B). One half was cultured alone for 24 hours, while the contralateral side was maintained in association with the underlying paraxial mesoderm. According to fate map analysis (Catala et al., 1996), this dorsal neural plate region corresponds to the prospective alar plate of the cervicothoracic region of the spinal cord (at the level of somites 19 to 22) and from previous experiments we know that it does not develop strong Pax6 expression when isolated in vitro (F. Pituello, unpublished observation). As shown in Fig. 3C, whereas the neural plate cultured alone displayed, at the most, a barely detectable level of Pax6 transcription, the presence of the paraxial mesoderm upregulated the level of Pax6 expression in more than 50% of the explants (17 positive explants out of 32 cocultures; Fig. 3D). This observation suggests that the differentiating paraxial mesoderm may not only participate in triggering the high level of transcription observed in region C cultures but, more interestingly, may be one of the elements responsible for the up regulation of Pax6 expression in the developing spinal cord in vivo.

Somitogenesis and the control of Pax6 upregulation in the cervical spinal cord
We next examined whether the rapid upregulation of Pax6 observed in the cervical spinal cord neighbouring each newly formed somite in stage 9-10 chicken embryos (see Fig. 1D) may be related to the paraxial mesoderm and particularly to somite formation. If so, Pax6 expression should be activated
prematurely in the neural plate by grafting the last somite formed from a donor embryo in place of nonsegmented mesoderm under the neural plate of a host embryo, in a zone that is not yet expressing detectable levels of the transcripts. Such heterotopic grafting experiments were performed on embryo cultures (see Materials and methods) operated on from the ventral side to preserve the contact with the dorsalizing non-neural ectoderm as well as with the ventralizing notochord. In host embryos (7- to 10-somite stage) we replaced a piece of the presomitic mesoderm with the last somite of a 7- to 10-somite-stage donor embryo (Fig. 4A). The graft was performed rostral to Hensen’s node in the prospective −III/−IV somite level. Embryos were then fixed 2.5 to 5 hours after grafting. In all the cases (n=7), the somite graft upregulated Pax6 expression in the adjacent neural plate (Fig. 4B). A strong level of Pax6 transcripts was observed above the grafted somite without caudal expansion of the gene expression in the neural plate posterior to the graft (Fig. 4B-D). In such conditions, Pax6 is activated in the entire lateral walls of the neuroepithelium adjacent to the graft, just excluding the floor plate area (data not shown), as has previously been described.

**Fig. 1.** Pax6 expression visualized by in situ hybridization in 1-day old cultures of region C. (A-C) Schematic representation showing the two regions of neural plate explanted for this study. (A) Diagram of a stage 10 embryo to show the rostrocaudal area of open neural plate explanted. (B) Schematic representation of cross sections of neural plate explants showing the tissue surrounding the neural plate. Floor plate precursor cells are probably already present at the ventral midline of the neural plate region R, C, structure of the two neural plate regions after dissection. (D-F) Pax6 expression visualized by in situ hybridization, (D) in stage 9 chicken embryos, (E) in neural plates explanted rostrally to Hensen’s node, (F) in neural plates explanted caudally to the node. Pax6 is detected in two bands of cells, one on each side of the ventral midline (E, arrow; dorsal view of the explant) in region R (E), whereas in region C a single continuous band of positive cells is seen (F; lateral view of explants). R, rostral; C, caudal; ec, ectoderm; fp, floor plate; Hn, Hensen’s node; nc, notochord; np, neural plate; pm, paraxial mesoderm; S, somite. Bar, 165 μm.

**Fig. 2.** Visualization of ventralizing cell types in 1-day-old neural plate cultures. Detection of HNF3β (A,C) and Shh (B,D) transcripts by in situ hybridization performed on 1-day-old neural plate cultures. All the region R explants are stained (A,B), whereas no staining is observed in region C explants (C,D). Bar, 165 μm.

**Fig. 3.** Cells expressing paraxis are present in cultures of the neural plate region C and the presence of paraxial mesoderm is sufficient to increase Pax6 expression. (A) In situ hybridization revealing the presence of paraxis transcripts in a 1-day-old culture containing four explants of neural plate region C. (B) Schematic representation of the dissection performed for C and D. Ld, dorsal half of the neural plate region located lateral to Hensen’s node; Pm, paraxial mesoderm. (C,D) After 1 day of differentiation in vitro, the explants cultured alone do not express Pax6 (C). In contrast, the presence of paraxial mesoderm in vitro results in the upregulation of Pax6 in at least 50% of the explants (D). Bar, 200 μm.
Somitogenesis and Pax6 activation for the wild-type gene expression in the spinal cord (see Introduction).

The activation of Pax6 transcription may be the result of the inhibition of a repressor, emanating from the underlying presomitic mesoderm, which maintains Pax6 at a low level until somites form. To verify this hypothesis, we performed a series of grafts in which a piece of presomitic mesoderm was replaced on one side by a somite (left side on Fig. 4C-D) and, on the contralateral side (right side on Fig. 4C-D), at the same anteroposterior level, by a Millipore membrane (n=4; Fig. 4C). We never observed an upregulation of Pax6 expression above the membrane while Pax6 was always activated above the somite (Fig. 4D). The upregulation of Pax6 thus appears as the consequence of a positive signal from the somite, rather than the suppression of a negative signal emanating from the presomitic mesoderm.

Since it has recently been demonstrated that low doses of SHH are sufficient to upregulate Pax6 expression (Ericson et al., 1997), one possibility may be that the somite has trapped enough SHH, emanating from the notochord, to upregulate Pax6 expression when grafted under the caudal neural plate. To block any SHH molecules that may have been trapped in the somite (which does not itself synthesize SHH), we preincubated the somite in the SHH-blocking antibody 5E1 (Ericson et al., 1996) prior grafting it. The optimum conditions for antibody fixation on living cells were determined using confocal analysis and quantification of fluorescence (see Materials and methods for details) in notochord explants incubated in SHH-blocking antibodies (Fig. 5A). Despite the absence of detectable immunostaining on the somite (Fig. 5B-C), the latter was grafted under the neural plate as previously described. As shown in Fig. 5D, the somite still activated Pax6 expression (n=6/6) under conditions in which any putative trapped SHH molecules would be blocked.

Two important points should be underlined: first, 2.5 hours of contact with the grafted somite were sufficient to produce an appreciable activation of Pax6 (Fig. 4). This period corresponds to the time necessary for the first new somite to become individualized in the operated host embryo, the following somites arising every 90 minutes. Second, the effect was observed with the last somite isolated from 10-somite stage embryos (n=2/2; data not shown) as well as with the second somite (s2) of 7-somite-stage embryos (n=2/2; data not shown).

Our observations suggest that somite formation could constitute a clock for the upregulation of Pax6, at least in the cervical spinal cord of stage 7-10-somite embryos. If this hypothesis is correct, by performing the reciprocal experiment, i.e., replacing the rostral part of the presomitic mesoderm, just before Pax6 activation, with younger presomitic mesoderm should delay the upregulation of the gene in the adjacent neural tube. Accordingly, we replaced the presomitic mesoderm at the level –I to –II of 7- to 8-somite-stage host embryos with younger presomitic mesoderm from level –IV to –V (Fig. 6A). After 3-3.5 hours of incubation a new somite had become individualized on the control side but, as expected, not on the operated side. Pax6 expression was normal on the control side, while it was undetectable or only faint on the side in contact

Fig. 4. A somite graft prematurely upregulates Pax6 in the neural plate. (A) Schematic representation showing the rostrocaudal level at which the presomitic mesoderm is replaced by a somite. (B) A somite graft (located between the two arrows) leads to a rapid upregulation of Pax6 above the somite, thus extending the caudal limit of Pax6 expression. (C,D) Examples of embryos with a somite inserted on one side (left) and a membrane on the contralateral (right) side (C). Pax6 transcription is upregulated over the somites whereas Pax6 is still absent above the membrane, as can be clearly seen after removing the membrane at a higher magnification (D). Bars, (C) 500 μm; (B,D) 200 μm.

Fig. 5. A pretreatment of the somite with SHH-blocking antibodies does not affect its ability to upregulate Pax6. (A–C) Confocal pictures of 5E1 antibody fixation in a notochordal explant (A; rostral is to the left) and in a somite (C), revealed using a rhodamine-conjugated anti-mouse IgG. All preparations were imaged at 5 μm depth. Note the absence of immunostaining in the somite (C); B is the corresponding phase contrast. (D) A somite (between arrows) preincubated in the blocking antibody against SHH (aSHH) still induces Pax6 upregulation. S, somite. Bars (A–C) 40 μm; (D) 200 μm.
with young presomitic mesoderm (n=7/7; Fig. 6B-C). Substituting a membrane for the presomitic mesoderm (n=1/1) produced the same effect (Fig. 6D-E), suggesting that removal of the somite from the vicinity of the neural tube is sufficient to delay Pax6 expression. This observation reinforces the model of an activation via the somite rather than that of an inhibition by the presomitic mesoderm. Interestingly, in some embryos incubated longer, Pax6 expression was also observed posterior to the graft where a new somite had individualized (Fig. 6D-E). The conclusion suggested by these experiments is that the activation of Pax6 at the cervical spinal cord level is related to somitogenesis.

In another set of experiments we sought to determine whether the somite was necessary for the maintenance of Pax6 expression. Accordingly, we replaced the somite at position +1 to +II, i.e., at a level where Pax6 is already expressed, with −IV to −V presomitic mesoderm (Fig. 6A) before analysing Pax6 expression in the adjacent neural tube. As shown in Fig. 6F-G, the presomitic graft had a drastic effect on Pax6 expression, which was strongly down-regulated on the operated side (n=4/4) within as little as 3 hours of incubation. This effect was not due to the presence of a potential inhibitor of Pax6 expression in the presomitic mesoderm since grafting a membrane in the position of somite +II also produced a down-regulation of Pax6 expression (n=3/4; data not shown). These results suggest that the somite is necessary not only for the activation but also for the maintenance of Pax6, at least for the 3 hours (a somite is individualized every 90 minutes) following Pax6 activation.

DISCUSSION

In the present study, we provide evidence that activation of Pax6 expression in the chicken cervical spinal cord may be controlled by the paraxial mesoderm in phase with somitogenesis. Strong levels of Pax6 transcription are observed in tissue isolated caudally to Hensen’s node in the absence of ventralizing cells but in the presence of differentiating paraxial mesoderm. While dorsal neural plate isolated at the level of Hensen’s node express barely detectable levels of Pax6 transcripts, coculturing them with the underlying paraxial mesoderm leads to high levels of Pax6 expression. In vivo, the transcription of Pax6 is sharply upregulated in the vicinity of the last formed somite (Goulding et al., 1993; Li et al., 1994), and grafting a somite caudally at the level of the presomitic mesoderm activates Pax6 prematurely in the neural plate. Conversely, replacing a piece of rostral presomitic mesoderm with the same tissue isolated more caudally delays the upregulation of Pax6 in the neural tube. Finally, replacing a somite with presomitic mesoderm down regulates Pax6 expression suggesting that the somite is also necessary for the maintenance of the expression of this gene at least in the initial steps.

SHH and Pax6 regulation

Several lines of evidence indicate that the initiation of Pax6 transcription is independent of the presence of ventralizing structures and signals. Mice carrying a null mutation of the gene coding for the transcription factor HNF3β fail to develop node, notochord and floor plate, but Pax6 is nevertheless transcribed in the neural tube (Ang and Rossant, 1994). A low level of Pax6 transcription has also been observed following notochord ablation in the chick embryo (Goulding et al., 1993). A comparable result has been obtained in mice homozygous for the mutation in the Shh gene (Chiang et al., 1996). In Shh−/− mice, it has been reported that Pax6 expression, normally high in the basal plate and weak in the alar plate, occurs uniformly throughout the neural tube at a reduced level characteristic of alar plate expression (Chiang et al., 1996).

However, it has recently been shown that the gradient in expression of Pax6 detected in vivo in the basal plate can be reconstructed in vitro by exposure of neural cells to different concentrations of SHH, low doses of the protein being associated with high levels of Pax6 expression (Ericson et al., 1997). Taken together, these observations suggest that the initiation of Pax6 transcription is independent of the ventralizing SHH-mediated signal, but that SHH play a major role in the regulation of the level of Pax6 expression.

In the present study, we observe that, (i) prospective neural plates isolated caudally to Hensen’s node and maintained in vitro display Pax6 expression in the absence of notochordal and floor plate cells; (ii) the presence of the notochord expressing SHH is not sufficient to upregulate and maintain Pax6 expression in the cervical spinal cord after removal of a somite. Consequently, we propose that mechanisms others than SHH signaling may be involved in regulating Pax6 expression in the neural tube.

Somitogenesis as a clock for Pax6 activation in the cervical spinal cord

The present observations suggest that signaling from the paraxial mesoderm is involved in regulating Pax6 expression in the developing neural tube. Coculturing the prospective alar plates with paraxial mesoderm results in a high level of Pax6 transcription in at least 50% of the alar plates, while alar plates cultured alone display only low levels of Pax6, or none at all. In vivo, the expression of Pax6 is detected in the developing cervical spinal cord in the vicinity of the last somite formed (Goulding et al., 1993; Li et al., 1994) and grafting the last somite of a stage 9-10 embryo, in place of unsegmented mesoderm, under the caudal neural plate of a host embryo of the same age is sufficient to activate Pax6 prematurely in the neural plate. Finally, Pax6 expression is delayed when the rostral part of the presomitic mesoderm is replaced with the same tissue isolated at the −IV to −V somite level. We therefore propose that the activation of Pax6 expression in the cervical spinal cord is under the control of the paraxial mesoderm, its inducing activity being related to somitogenesis.

Here we observe that the time of contact sufficient to activate Pax6 expression after grafting a somite is comparable to the time necessary for the individualization of a single somite in the host embryo. Recently, molecular evidence has been provided that a developmental clock may be linked to somitogenesis of the paraxial mesoderm (Palmeirim et al., 1997). The developing spinal cord has no obvious anteroposterior landmarks, but genes such as Pax6 are activated at precise times and locations along the rostrocaudal axis and here we show that such an activation correlates with somitogenesis. It is therefore, tempting to speculate that, at least in some regions of the developing spinal cord,
somitogenesis may be used as a clock to activate specific genes in a temporally and spatially appropriate manner.

After closure of the neural tube over the sinus rhomboidalis, the caudal limit of Pax6 expression expands further caudally than the last individualized somite. This observation could reflect the presence of Pax6-inducing activity in the presomitotic mesoderm. We have verified this point by isolating the presomitotic mesoderm at level III–IV in 19-somite-stage embryos. This stage was chosen after taking into account the fact that a high level of Pax6 transcripts should be present in the neural tube facing the presomitotic mesoderm concerned. The latter was grafted under the neural plate of younger embryos, as described for a somite with inducing activity (Fig. 4A). Under such conditions, we did not observe Pax6 activation on top of the graft (n=4/4; data not shown). Therefore, in more caudal regions of the developing neural tube, the upregulation of Pax6 facing the unsegmented paraxial mesoderm involves a mechanism different than the one described here for the cervical spinal cord. In another situation, i.e. the loss of Noggin function in murine mutant (McMahon et al., 1998), Pax6 is present down to the hindlimb but absent from the caudal spinal cord. Hence, along the rostrocaudal axis, different strategies are probably used to regulate Pax6 expression in the developing spinal cord.

There is increasing evidence that the paraxial mesoderm plays a major role in controlling the patterning of the developing neural plate. In the zebrafish embryo, grafting non-axial mesendoderm near the forebrain results in a posteriorization and the development of ectopic hindbrain-like structure (Woo and Fraser, 1997). In avian embryos, it has recently been shown that, early in development, the paraxial mesoderm may participate in the caudalization of the neural plate (Bang et al., 1997; Muhr et al., 1997), a property also attributed to late Hensen’s node (Kintner and Dodd, 1991; Bang et al., 1997; Henrique et al., 1997). During normal development of the chicken embryo, such a caudalizing activity of the paraxial mesoderm is detected for a limited period, from stage 4 to 9, and over this period the activity becomes restricted to the paraxial mesoderm adjacent to the neural plate at caudal levels (Muhr et al., 1997). This profile of caudalizing activity strengthens our suggestion that the effect on Pax6 activation we describe here is different from the caudalizing activity, since a somite isolated from a stage 10 embryo, which no longer displays caudalizing activity (Muhr et al., 1997), is able to activate Pax6 expression prematurely in the neural plate.

It has also been shown that a somite-derived signal has the ability to influence the programme of Hox expression and induce posteriorization in anterior hindbrain (Itasaki et al., 1996; Grapin-Botton et al., 1997). Transposing a set of somites from different anteroposterior levels adjacent to the anterior hindbrain of stage-10 host embryos leads to a reprogramming of Hox expression as well as that of other segmentally restricted genes. There is a rostrocaudal difference in the ability of somites to alter Hox expression. For example at stage 10 in the chick embryo, only somites posterior to the 6th reproducibly induce posterior Hox genes. We observe that the second somite of a stage 10 embryo is still able to activate Pax6 expression after grafting under the caudal neural plate (data not shown); the mechanism involved in Pax6 activation is therefore probably different from that involved in the reprogramming of Hox expression after rhombomere transposition. Neural cells of the spinal cord have also been shown to be plastic at the time of neural tube closure and sensitive to signals from the paraxial mesoderm; such signals from the paraxial mesoderm may control the identity of motor neuron columnar subtypes differentiating at different segmental levels of the spinal cord. Rostrocaudal inversion of the lumbosacral neural tube (Matise and Lance-Jones, 1996) or interchange between thoracic and brachial spinal cord levels (Ensini et al., 1998) results in a reprogramming of motor neuron subtypes, which occurs independently of the notochord (Matise and Lance-Jones, 1996). It depends however upon the paraxial mesoderm, since switching the paraxial mesoderm between thoracic and brachial levels modifies the motor neuron identity (Ensini et al., 1998).

In view of our present data, the observation that signals from the somite may restrict the rostrocaudal movements of cells in the ventrolateral neural tube of the trunk is particularly interesting (Stern et al., 1991). In HH stage 9-14 chick embryos, clones derived from single cells stained in the neural tube in situ at the level of the most recently segmented somite, tend to expand along the mediolateral axis, while clones derived from neural cells opposite to the unsegmented mesoderm tend to expand along the rostrocaudal axis. Furthermore, replacing the somite with caudal presomitic mesoderm endows the neural cells with a migratory behaviour similar to that observed in caudal neural plate regions. In the present study, we observe defective Pax6 activation in comparable experiments. In the forebrain of Sey/Sey mutant mice, the absence of Pax6 function has been associated with defects in the aggregative behaviour of cortical and striatal cells, alteration of adhesive molecules and defects in boundary formation (Stoykova et al., 1997). The observations that the restriction of cell movements and the appearance of Pax6 in the spinal cord may be concomitant and that both events may be controlled via somitogenesis, together with the possible relationship between Pax6 and the aggregative behavior of neural precursor cells, suggest that the restriction of cell movement in the spinal cord may be correlated with the appearance of Pax6.

A model for the establishment of the dorsoventral polarity in the cervical spinal cord

Taking into account current data concerning the regulation of Pax gene expression, we propose the following model for the establishment of dorsoventral polarity in the caudal neural plate of a stage 9-10 chicken embryo. The neural plate positioned caudally to Hensen’s node displays a Pax3-on, Pax6-off state (Fig. 7A; Goulding et al., 1993; Liem et al., 1995; Bang et al., 1997). Pax3 expression has probably been initiated earlier in the neural plate by posteriorizing signals produced by late Hensen’s node and non-axial mesoderm (Bang et al., 1997). Rostrocaudally to Hensen’s node, Pax3 will be progressively extinguished from the lateral walls of the neural plate to become restricted to the neural folds (Fig. 7B,C; Goulding et al., 1993; Liem et al., 1995). It has been proposed that the restriction of the Pax3 expression domain to the dorsal part of the neural tube is controlled by the opposing action of the dorsalizing (BMPs-mediated) and ventralizing (SHH-mediated) signals (Goulding et al., 1993; Liem et al., 1995). At this stage of development, BMPs are expressed in the
dorsalizing non-neural ectoderm (Liem et al., 1995) and SHH protein is detected in the notochord but not yet in the presumptive floor plate area, where the protein will be detected only more rostrally (Martí et al., 1995). The down-regulation of \(Pax3\) observed in the prospective basal plate is proposed to be the first step of the SHH-dependent programme and this first step of \(Pax3\) off seems to be a prerequisite for ventral cell type differentiation (Ericson et al., 1996; Tremblay et al., 1996). We could therefore suppose that this phase of \(Pax3\) down-regulation would be sufficient to allow \(Pax6\) expression. However, several observations indicate that it is not the case. In transgenic mice misexpressing \(Pax3\) in the basal plate, \(Pax6\) expression is not affected (Tremblay et al., 1996). Furthermore, the expression domains of these two genes are not mutually exclusive, since later on in the alar plate they clearly overlap (Goulding et al., 1993). Therefore, the activation of \(Pax6\) involves mechanisms other than the switching off of \(Pax3\). Here we demonstrate that the activation of \(Pax6\) at the cervical spinal cord level may involve the paraxial mesoderm in phase with somitogenesis (Fig. 7D).

Two hypotheses may thus be proposed: either a negative signal from the early paraxial mesoderm inhibits \(Pax6\) expression detected only more rostrally (Martí et al., 1995). The down-regulation of \(Pax3\) observed in the prospective basal plate is proposed to be the first step of the SHH-dependent programme and this first step of \(Pax3\) off seems to be a prerequisite for ventral cell type differentiation (Ericson et al., 1996; Tremblay et al., 1996). We could therefore suppose that this phase of \(Pax3\) down-regulation would be sufficient to allow \(Pax6\) expression. However, several observations indicate that it is not the case. In transgenic mice misexpressing \(Pax3\) in the basal plate, \(Pax6\) expression is not affected (Tremblay et al., 1996). Furthermore, the expression domains of these two genes are not mutually exclusive, since later on in the alar plate they clearly overlap (Goulding et al., 1993). Therefore, the activation of \(Pax6\) involves mechanisms other than the switching off of \(Pax3\). Here we demonstrate that the activation of \(Pax6\) at the cervical spinal cord level may involve the paraxial mesoderm in phase with somitogenesis (Fig. 7D).
until the formation of the somite, or the activation of Pax6 in the neural tube is the result of the appearance of an activator in the individualizing somite. The observation that replacing a piece of caudal mesoderm by a somite leads to a premature activation of Pax6 while replacing it by a neutral membrane does not trigger Pax6 expression suggests that the somite is an activator of Pax6 expression. This idea is reinforced by the complementary experiment in which the rostral presomitic mesoderm or a somite is replaced either by caudal presomitotic mesoderm or by a membrane; in both cases, Pax6 expression is absent, which suggests that removal of the somite is sufficient to block the normal acquisition of Pax6 expression. Interestingly, we also show that the presence of the somite is necessary for the maintenance of Pax6 expression. All together, these data argue in favour of a model in which Pax6 is activated in the cervical spinal cord via a positive signal from the somite, this signal being maintained at least for the next few hours to stabilize the gene expression. The nature of the signaling molecule mediating Pax6 upregulation remains unknown. The fact that a preincubation of the somite in blocking anti-SHH antibodies does not abolish the activity of the somite suggests that the factor is not SHH, even if this molecule is well known for its ability to upregulate Pax6 expression (Ericson et al., 1997).

Finally, in a further step, the level of Pax6 expression may be modulated via a gradient of SHH (Fig. 7E; Ericson et al., 1997). It has been shown that a gradient established by SHH diffusing in the basal plate will give rise to a gradient of Pax6 expression in the ventral progenitor cells, allowing the emergence of different subpopulations of progenitors and subsequently of different subpopulations of neurons. The level of Pax6 expression seems therefore a critical point in the genesis of distinct subpopulations of neurons in the spinal cord, particularly at the cervical level, where the absence of Pax6 function is accompanied by defects of ventral interneuron differentiation and mis specification of motor neurons (Burrill et al., 1997; Ericson et al., 1997; Osumi et al., 1997). Here, we show that one of the strategies that the embryo may use for fine regulation is somitogenesis.

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