cSox3 Expression and Neurogenesis in the Epibranchial Placodes

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Epibranchial placodes are local thickenings of the surface ectoderm, which give rise to sensory neurons of the distal cranial ganglia. The development of these placodes has remained unclear due to the lack of any definitive marker for these structures. We show here that the chick transcription factor, cSox3, is expressed in four lateral patches at the rostral edge of the epibranchial arches and that these mark the epibranchial placodes. These patches of cSox3 expression arise by gradual thinning from broader areas of cSox3 expression with concomitant loss of cSox3 in nonplacodal regions. Cells leaving the epithelial placodes as they initiate neurogenesis, lose cSox3 expression and sequentially express Ngn1, NeuroD, NeuroM, and Phox2a, but do not express Ngn2. This is in contrast to studies in the mouse where it is Ngn2, rather than Ngn1, that is predominantly expressed in epibranchial-derived neuroblasts. Overexpression of cSox3 interferes with normal neuroblast migration and results in changes in ectodermal morphology. Thus, cSox3 provides a useful tool for the study of placode formation, and loss of cSox3 expression appears to be a necessary event in normal neurogenesis from the epibranchial placodes.   © 2001 Academic Press

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INTRODUCTION

The entire nervous system of vertebrates is derived from the ectoderm of the early embryo. Although many aspects of the molecular mechanisms involved in CNS and neural crest development have been elucidated, relatively little is known in relation to the neurogenic placodes. Placodes have been defined as local areas of thickened ectoderm which give rise to both neuronal and non-neuronal structures (the lens of the eye for example). In amniotes, there are four categories of neurogenic placode: the olfactory, otic, trigeminal, and epibranchial placodes. Both the olfactory and otic placodes give rise to neuronal and non-neuronal components. The trigeminal and epibranchial placodes contribute exclusively neurons, producing the sensory neurons of distal cranial ganglia, the glial cells and other neurons being derived from neural crest cells. The epibranchial placodes are therefore an important component of the developing peripheral nervous system and exhibit unique changes in morphology and biology before

eventually disappearing after undergoing neurogenesis. Despite this, little is known about their formation and the control of their subsequent development, largely due to the lack of appropriate markers of early placodes.

The placodes described above are formed in a variety of ways and all arise within the first few days of development in chicks. The lens placode, the only entirely non-neurogenic placode, is induced by the underlying brain tissue that will form the retina. The olfactory and otic placodes are formed from ectoderm directly lateral to the neural fold epithelium. FGF3 has recently been shown to induce ectopic otic epithelium capable of generating neurons (Vendrell *et al.,* 2000). Formation of the epibranchial placodes over the second and third days of development is believed to result from induction by nearby tissues, either underlying mesoderm derived from cranial neural crest or endoderm. However, recent evidence from Begbie *et al.* (1999) showed that epibranchial endoderm is capable of inducing neurogenesis from surface ectoderm, even in the absence of neural crest.

The earliest events in placode development are poorly understood at the molecular level. Until recently, no markers of undifferentiated placodes have been described, prior

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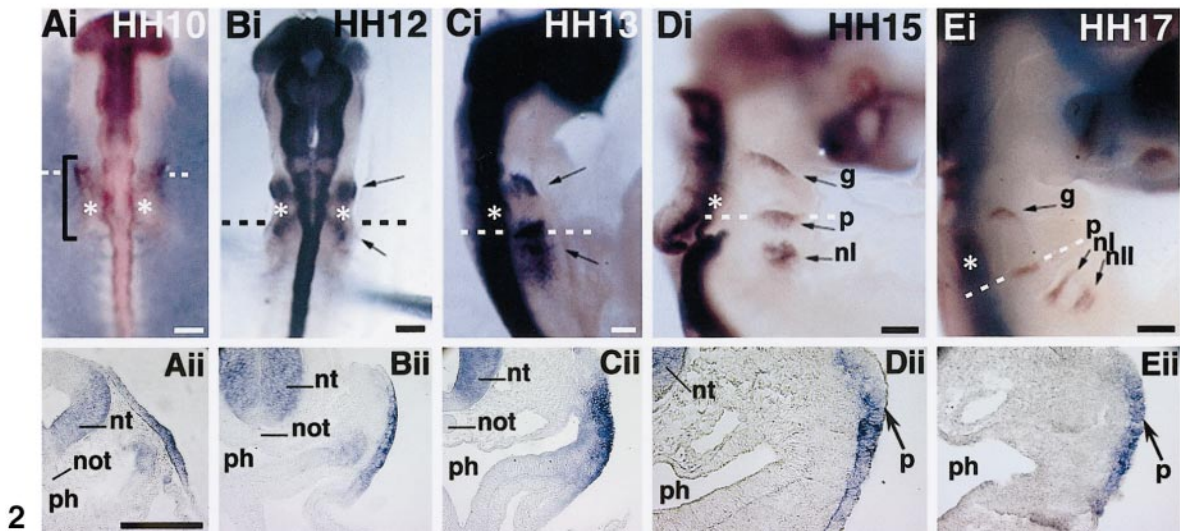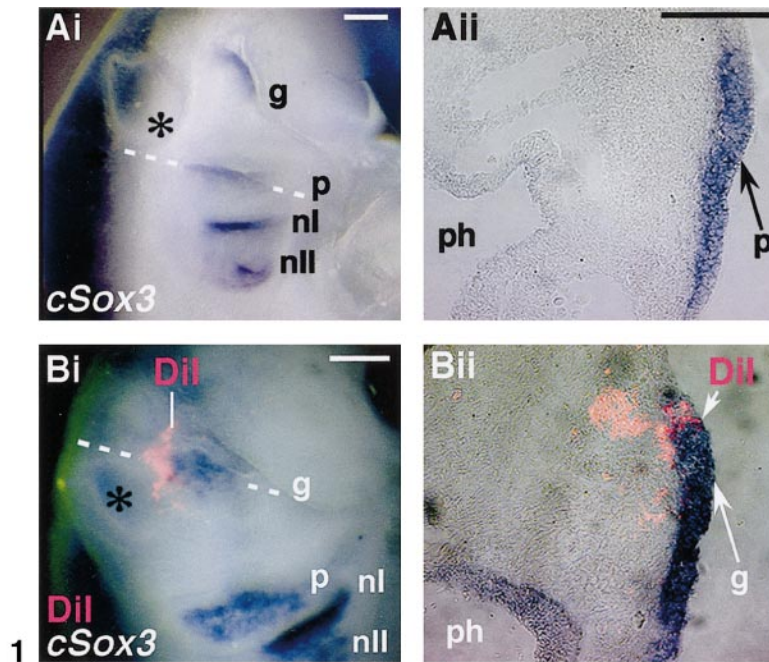
FIG. 1. cSox3 expression in epibranchial placodes at HH stage 18 analyzed by *in situ* hybridization and combined with DiI labeling. (Ai–Bi) Whole-mount embryos, rostral is up and dorsal is to the left. (Ai) cSox3 expression detected as four patches at the posterior aspect of each epibranchial cleft. (Aii) Transverse section through embryo in (Ai) (dotted line) showing expression of cSox3 in the thickened ectoderm of the first post otic placode, the petrosal. (Bi) DiI labeling (red) of the surface ectoderm of the geniculate placode carried out at HH stage 15 and analyzed at HH stage 18 then combined with *in situ* hybridization for cSox3 expression (blue). (Bii) Transverse section through embryo in (Bi) (dotted line) showing migrating DiI-labeled cells from cSox3 expression area and DiI-labeled cells on the surface ectoderm adjacent to cSox3 expression domain. Asterisk indicates the otic vesicle. G, geniculate placode; p, petrosal placode; nI, first nodose placode; nII, second nodose placode; ph, pharynx. Scale bar, approximately 100 μm.

FIG. 2. cSox3 expression analyzed by *in situ* hybridization in the developing epibranchial region. HH stage of the embryos is shown in top right corner of each panel. Top row (Ai–Ei) shows whole-mount embryos, rostral is up, caudal is to the bottom (Ai–Bi), dorsal is to the left (Ci–Ei). Bottom row (Aii–Eii) shows transverse sections through embryos in top row (dotted lines), just caudal to the otic pit or vesicle (asterisk), (except in Aii, section is just rostral to the otic pit). (Ai) cSox3 expression at HH stage 10 appears as a large patch around the otic pit (bracket). (Bi–Ci) cSox3 expression at HH stage 12–13 showing expression becoming more pronounced into two large patches (arrows). (Di) cSox3 expression at HH stage 15, the expression appears as three patches (arrows), note that the caudal patch in HH stage 13 has resolved into two restricted patches at HH stage 15. (Ei) cSox3 expression at HH stage 17, cSox3 expression appears as four restricted patches (arrows). Note cSox3 expression in the thickened ectoderm (Aii–Eii). G, geniculate placode; p, petrosal placode; nI, first nodose placode; nII, second nodose placode; nt, neural tube; not, notochord; ph, pharynx. Scale bar, approximately 100 μm.

to the appearance of cells undergoing neuronal development (Webb and Noden, 1993). However, members of the Pax gene family of transcription factors have now been shown to be expressed in placodes at an early stage in their development. These studies have shown cPax6 to be expressed in lens and olfactory placodes, while cPax2 is expressed in the otic and epibranchial placodes (Grindley *et al.*, 1995; Baker and Bronner-Fraser, 2000) and cPax3 expression is restricted to the ophthalmic trigeminal placode (Baker *et al.*, 1999).

cSox3 is expressed in all the epibranchial placodes and this expression arises from a larger region of ectoderm from which the placodes gradually form by thinning of the surrounding nonplacodal ectoderm (Ishii *et al.*, 2001). We demonstrate that it is from these final patches of cSox3 expression that cells migrate inwards and undergo neurogenesis. We show the sequential expression of several basic helix–loop–helix (bHLH) transcription factors as cells migrate from the placodes and we demonstrate a number of differences from the gene expression described during mouse development (Fode *et al.*, 1998). In particular, chick neurogenin2 (cNgn2) is expressed exclusively in the trigeminal placode and chick neurogenin1 (cNgn1) in the epibranchial placodes, the reverse of the situation in mice (Fode *et al.*, 1998; Ma *et al.*, 1998, 1999). Overexpression of cSox3 interferes with normal migration of cells from the placodes, suggesting that loss of cSox3 is a necessary step in placodal neurogenesis. Ectopic cSox3 expression also results in abnormal morphological changes in the surface ectoderm, suggesting a role in control of epithelial structure.

MATERIALS AND METHODS

Embryos

White Leghorn chicken eggs were incubated at 39°C. Embryos were staged according to Hamburger and Hamilton (1951). For all types of operations, a small window was made in the egg. Diluted Indian ink (Rotring, GmbH, Black, D-2510) in PBS was injected beneath the blastoderm to visualize the embryo.

DiI Labeling

CellTracker CM-DiI (Molecular Probes) was dissolved in dimethylformamide at a final concentration of 0.5%. DiI was warmed to 40°C before use then centrifuged for 1 min. A very fine glass microcapillary (50 μm in diameter) was filled with the DiI and a small bolus of the dye was then applied by a gentle air pressure to the desired epibranchial placodal region. The exact position of the labeling was checked under a fluorescent microscope and photographed using a CCD camera (DC-330- DAGE-MIT). The eggs were then sealed and allowed to develop for 24 h. DiI labeling was examined for any changes after incubation and photographed under a fluorescent microscope. Embryos were fixed overnight in 4% paraformaldehyde/PBS at 4°C. Embryos were then processed for whole-mount *in situ* hybridization.

In Ovo Electroporation

In ovo electroporation (EP) was used in the current study to obtain ectopic expression of cSox3 and GFP in the epibranchial placodes. The full coding sequence of cSox3 was subcloned into the pEGFP-C1 vector (Clontech) to make the pEGFP-C1/cSox3 expression construct. The pEGFP-C1 expression vector was used to overexpress GFP as a control. All constructs were dissolved at 3 μg/μl in PBS/1 mM MgCl$_2$. A modified method of Momose *et al.* (1999) was applied. A small hole was made in the vitelline membrane and the amniotic membranes were gently removed, exposing the epibranchial region. In order to avoid direct contact of the electrodes with the blood vessels and embryonic membranes, a piece of shell membrane was placed on each side of the embryo such that one piece covered the heart. A platinum electrode (0.5 mm in diameter) and tungsten cathode (0.25 mm) were placed on the shell membranes 3 mm apart, sandwiching the embryo. DNA mixed with red food coloring (Supercook, U.K.) was injected onto the surface of the epibranchial placodes by a gentle air pressure through a fine microcapillary. Square pulses (three 23-V pulses, 25-ms pulse length, 150-ms interval between pulses) were generated by an electroporator Square CUY-21. (BEX CO., LTD, Tokiwasaiensu, Japan). Resistance between the electrodes was maintained at 1.2–2 KΩ. PBS was added before and immediately after each operation. After electroporation, eggs were sealed and incubated for 24–48 h. Embryos were then examined under the fluorescent microscope for green fluorescence signals. Embryos with good GFP fluorescence were collected and fixed overnight in 4% paraformaldehyde/PBS at 4°C.

In Situ Hybridization

Single-stranded riboprobes were prepared incorporating either digoxigenin–UTP or fluorescein–UTP (Roche), according to the manufacturer's recommendation of the Riboprobe Gemini System (Promega Ltd.). The full length of cSox3, cNgn1, and cNgn2 (gifts from Dr. David Anderson), mNgn1 and mNgn2 (gifts from Dr. François Guillemot), cNeuroD and cNeuroM (gifts from Dr. Marc Ballivet), and cPhox2a (gift from Dr. Jean-François Brunet), were used to make anti-sense riboprobes. For single nonradioactive *in situ* hybridization, the method described by Cheung *et al.* (2000) was used. To detect the endogenous expression of two genes on whole mount, the method described by Cheng *et al.* (2000) for double *in situ* hybridization was applied. For this method, two probes labeled with fluorescein–UTP and digoxigenin–UTP were hybridized simultaneously. The expression of the first gene was detected by using Fast Red TR/Naphthol AS-MX (Sigma Fast) followed by detection of the second gene in blue by using NBT/BCIP (Roche). The same protocol was applied to detect the ectopic expression of an overexpressed gene and the endogenous expression of another gene. Ectopic expression was detected first by whole-mount *in situ* hybridization with Fast Red, then embryos were processed for frozen sectioning. Sections were then processed for immunostaining to detect the second probe using NBT/BCIP.

Immunohistochemistry

Anti-neurofilament monoclonal antibody (3A10, developed by Tom Jessell and Jane Dodd and obtained from the Developmental Studies Hybridoma Bank), anti-HuC/D, and anti-GFP polycolonal antibody (Clontech) were used as described by Rex *et al.* (1997). All treatments were carried out at room temperature and the antibody dilutions were prepared in 5% sheep serum/PBS. Briefly, sections

were blocked with 2% blocking reagent (Roche)/Maleic acid buffer for 75 min. This was followed by incubation in primary antibody (1 h at 1:3 dilution for 3A10 and at 1:100 dilution for anti-GFP and anti-HuC/D at room temperature). After washing with PBS, sections were treated with biotinylated anti-mouse universal secondary antibody (Vector Laboratories) for 1 h. Following a brief rinse with PBS, sections were treated with alkaline phosphatase-conjugated streptavidin (Vector Laboratories) at 1:500 in PBS for 30 min followed by a brief rinse in PBS. Sections were incubated in Fast Red (Sigma, prepared according to supplier's instruction) and the color was allowed to develop for 15–120 min. Sections were mounted in Mowiol (Calbiochem).

RESULTS

cSox3 Is Expressed in the Epibranchial Placodes

The epibranchial placodes are most evident during the third day of chick development (HH stage 18–20). At these stages, four patches of cSox3 expression were observed, restricted to small regions on the caudal aspect of each branchial cleft (Fig. 1Ai). The location of these patches approximately matches the regions that have been identified previously as the neurogenic epibranchial placodes that give rise to sensory neurons of the geniculate, petrosal, and nodose ganglia (D'Amico-Martel and Noden, 1983). The neurogenic placodes are, by definition, thickened areas of ectoderm from which cells delaminate, migrate, and undergo neurogenesis. We therefore analyzed the morphology of the cSox3-expressing cells in these patches in sections from embryos following whole-mount *in situ* hybridization. This clearly showed that the patches localized to areas of thickened epithelium (Fig. 1Aii). In order to establish that the patches of cSox3 expression were indeed the placodes from which cells migrated to the cranial ganglia, we adopted the following strategy. The regions in which we had identified cSox3 expression were labeled with the lipophilic dye, DiI, at HH stage 15 and allowed to develop for 16–24 h. It was anticipated that those labeled cells that lay outside of the placodes would remain on the surface while those that were in the placodes would migrate inwards. Combination of this analysis with *in situ* hybridization for expression of cSox3 after the labeled cells had been allowed to migrate, established the relationship between cSox3 expression and the region of cell migration. As shown in Figs. 1Bi and 1Bii, the region of cSox3 expression in the ectoderm overlapped the region where DiI-labeled cells had migrated inwards with concomitant loss of cSox3 expression. DiI-labeled cells immediately adjacent to the patches of cSox3 expression remained at the surface. Thus, the patches of cSox3 expression were both local areas of thickened ectoderm and regions from which ectodermal cells delaminated and migrated inwards, confirming their identity as placodes.

The identification of two distinct patches of cSox3 expression at the third and fourth clefts suggests that two placodes contribute to the nodose ganglion. This is an issue that has previously been confused in the literature, with authors describing either three (Le Douarin *et al.,* 1986;

Begbie *et al.,* 1999; Graham and Begbie, 2000) or four (D'Amico-Martel and Noden, 1983; Webb and Noden, 1993) epibranchial placodes in amniotes. Our data confirm that, in chick at least, like anamniotes, neurons of the nodose ganglion arise from more than one discrete placode.

In order to gain further insight into the manner in which the placodes are actually formed, we studied the appearance of the patches of cSox3-positive ectoderm over the first two days of chick development. cSox3 expression in the region of ectoderm which later covers the epibranchial arches could be distinguished as early as HH stage 6, when cSox3 expression was quite general throughout the ectoderm (Rex *et al.,* 1997b). By HH stage 8, ectodermal cSox3 expression outside of the CNS was largely lost, although some was retained in the region where the otic pit and epibranchial arches would form (Rex *et al.,* 1997b). By HH stage 10, cSox3 expression became restricted to a large patch of ectoderm around (and including) the developing otic pit, encompassing the region where presumptive epibranchial ectoderm is located (Fig. 2Ai) (Groves and Bronner-Fraser, 2000). Transverse sections of embryos following whole-mount *in situ* hybridization for cSox3 expression showed that this entire region exhibited a thick columnar epithelial morphology (Fig. 2Aii). Between HH stages 10 and 17, the region of cSox3-positive ectoderm became gradually restricted to more lateral patches (Figs. 2Bi–2Ei). Expression resolved first into two patches (HH stage 11–12), one rostral and one caudal to the otic vesicle (Fig. 2Bi). At HH stage 13, the caudal patch resolved into a larger patch (Fig. 2Ci). At HH stage 14–15, the larger caudal patch was seen to resolve into two patches (Fig. 2Di), and the more caudal of these was later divided again (from HH stage 17) (Fig. 2Ei). During HH stage 18–20, the patches of cSox3-positive expression became restricted to a small region caudal to each branchial cleft, the region of the presumptive placodes. Throughout these stages, as cSox3 expression became restricted, the columnar epithelium morphology became correspondingly restricted, as evident from transverse sections (Figs. 2Aii–2Eii). Thus, the placodes arise from gradual loss of columnar, cSox3-positive ectoderm, these two features always corresponding closely. It is interesting in this context that the only nonthickened neurogenic "placode," which gives rise to the trigeminal ganglion, does not express significant levels of cSox3.

From HH stage 21, cSox3 expression decreased, was detected at very low levels by HH stage 23, and was barely detectable by HH stage 24 when there was a corresponding loss of thickened ectoderm in these regions (data not shown).

Sequential Expression of Transcription Factor-Encoding Genes in Cells Migrating from the Epibranchial Placodes

Recently, a number of genes have been shown to be expressed sequentially as cells undergo neurogenesis from the epibranchial placodes in mice (Fode *et al.,* 1998). Among these are several that encode transcription factors of the
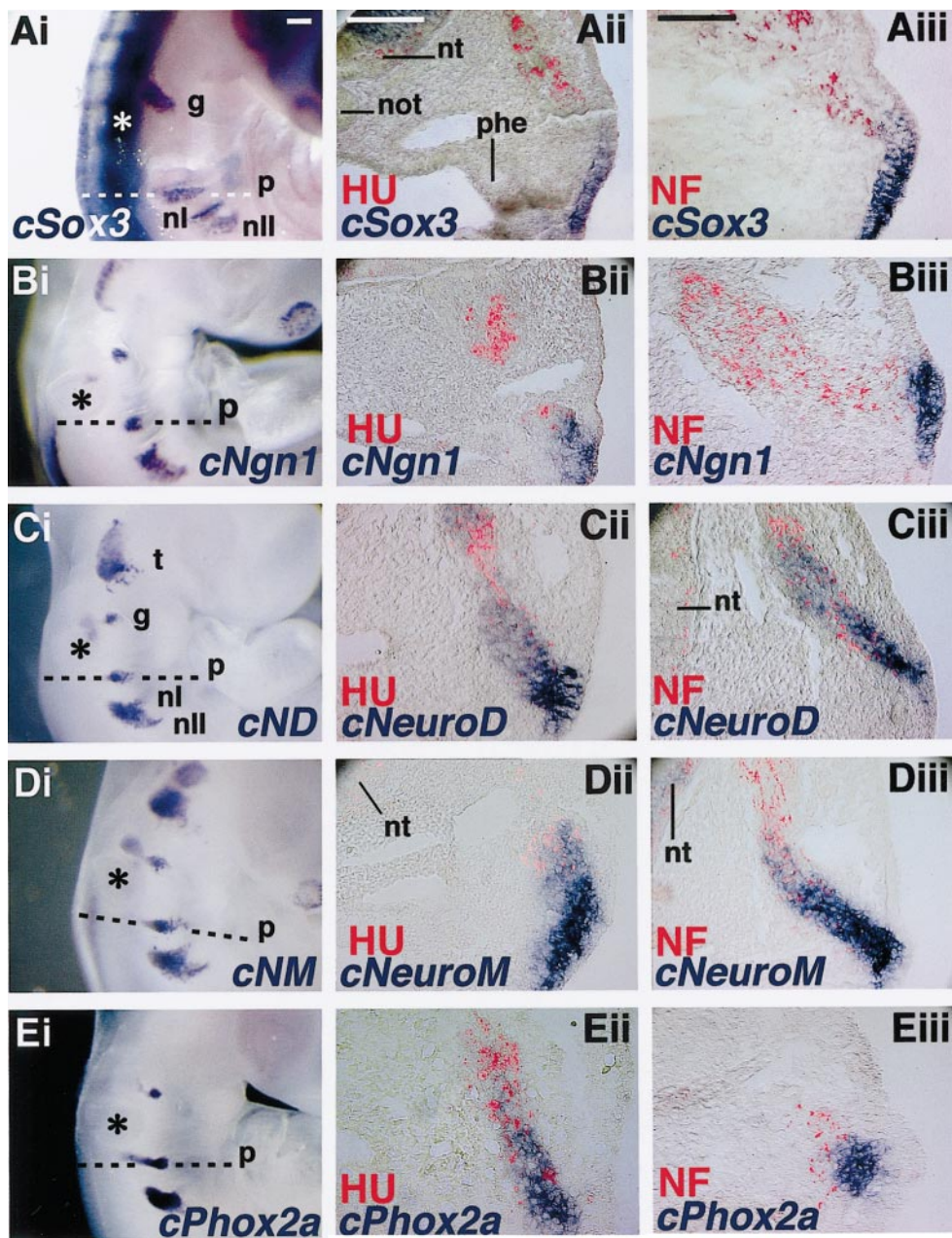
FIG. 3. Expression of cSox3, cNeurogenin1 (cNgn1), cNeuroD (cND), cNeuroM (cNM), and cPhox2a (*in situ* hybridization in blue) in relation to Hu and neurofilament (NF) proteins (immunohistochemical labeling in red) at HH stage 18. The first column (Ai–Ei) shows whole-mount embryos processed for whole-mount *in situ* hybridization for cSox3, cNgn1, cNeuroD, cNeuroM, and cPhox2a expression, respectively, in the epibranchial and trigeminal placodes (rostral is up and dorsal is to the left). The second column (Aii–Eii) and third column (Aiii–Eiii) show transverse sections through the petrosal placode from embryos in (Ai–Ei) (dotted lines) stained in red for Hu and NF, respectively. The asterisk indicates the otic vesicle. t, trigeminal placode; g, geniculate placode; p, petrosal placode; nI, first nodose placode; nII, second nodose placode; nt, neural tube; not, notochord; phe, pharyngeal endoderm. Scale bar, approximately 100 μm.

bHLH class. However, the detailed relationship between the expression of these genes in placodal neurogenesis has not been described in chick. We therefore set out to establish how the expression of these genes related to the region and period of expression of cSox3. This would also confirm whether the patches of cSox3 expression were indeed regions of neurogenesis. In order to relate the expression of these genes to neuronal differentiation, we compared their expression with the presence of the Hu and neurofilament (NF) proteins, regarded as markers of early and late neuronal
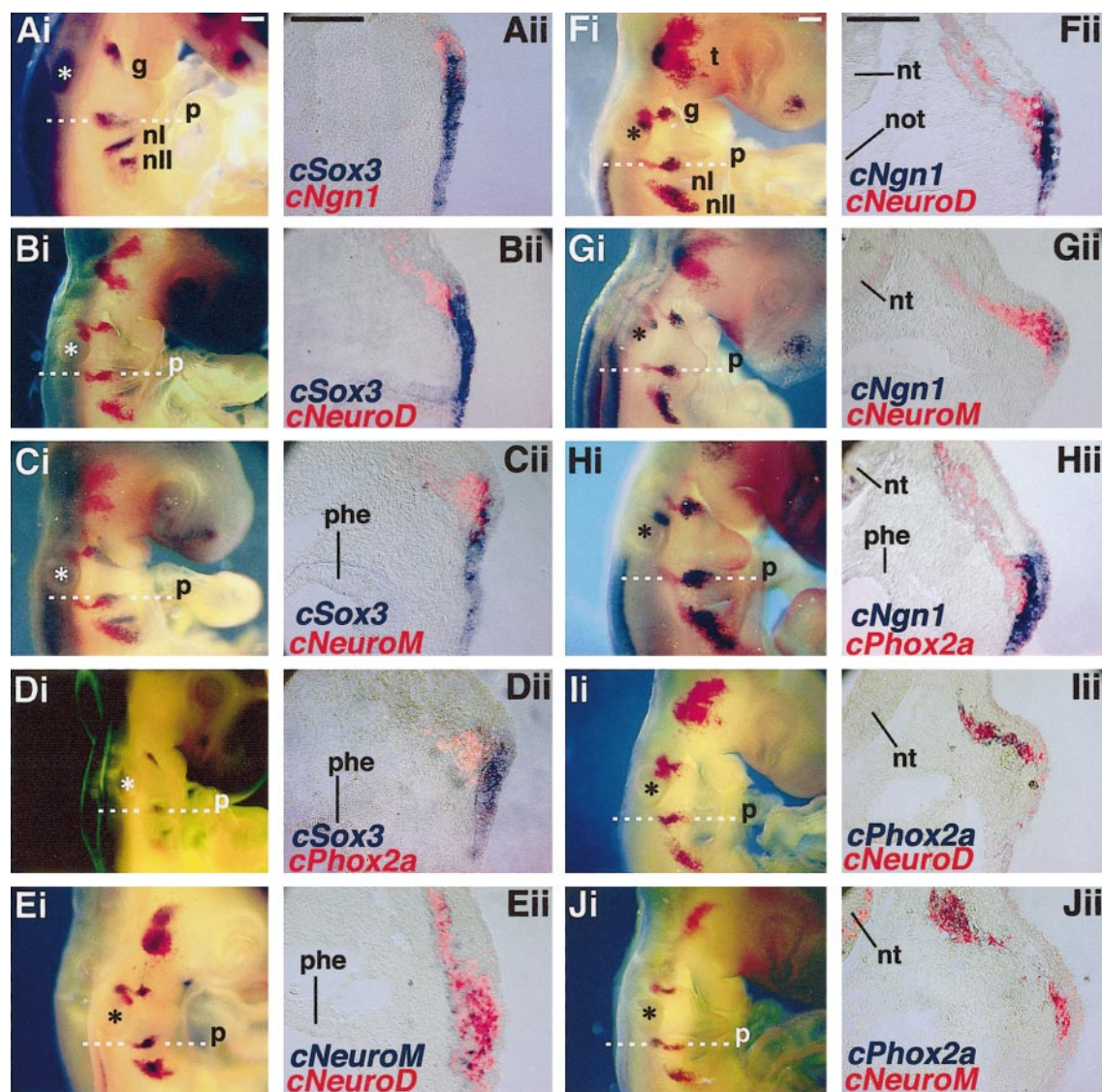
FIG. 4. Double *in situ* hybridization for transcription factor-encoding genes showing their expression in the epibranchial and trigeminal placodes at HH stage 18. First and third columns (Ai–Ji) show embryos after double whole-mount *in situ* hybridization; rostral is up and dorsal is to the left. Second and fourth columns (Aii–Jii) show sections through the petrosal placode of the embryos in the first and third columns (dotted lines). (Ai–Di) cSox3 expression detected in blue and stained for cNeurogenin1 (cNgn1), cNeuroD, cNeuroM, and cPhox2a expression, respectively, in red. (Ei) cNeuroM expression detected in blue and stained for cNeuroD expression in red. (Fi–Hi) cNgn1 expression detected in blue and stained for cNeuroD, cNeuroM, and cPhox2a expression, respectively, in red. (Ii, Ji) cPhox2a expression detected in blue and stained for cNeuroD and cNeuroM expression, respectively, in red. t, trigeminal placode; g, geniculate placode; p, petrosal placode; nI, first nodose placode; nII, second nodose placode; nt, neural tube; not, notochord; phe, pharyngeal endoderm. Scale bar, approximately 100 μm.

differentiation, respectively, based on the central nervous system. Data are presented for the second epibranchial placode, which gives rise to the petrosal ganglion. The observations described were, however, also seen in the other placodes unless otherwise stated. Both Hu and NF proteins were detected in cells after delamination from the placodes, overlapping the cell population that expressed cNgn1, cNeuroD, cNeuroM, and cPhox2a, and were main-

tained later in ganglion development (Fig. 3). NF appeared in migrating cells nearer to their placode of origin (Figs. 3Aiii–3Eiii), while Hu was only detected a little further away from the placodes (Figs. 3Aii–3Eii), suggesting that NF expression precedes Hu in these placodal-derived cells. However, neither protein was detected in cells that still expressed cSox3. We found that cNgn1 and cNeuroD were expressed in a large number of cells in the placodal ecto-

derm prior to delamination (Figs. 3Bii and 3Cii), while cNeuroM was expressed in very few cells still located in the thickened ectoderm (Fig. 3Dii). cPhox2a was only expressed in cells no longer in the surface ectoderm (Figs. 3Eii and 3Eiii), suggesting that it was only transcriptionally active after the cells had delaminated. The maintenance of expression of each gene as cells moved to their final location in the ganglion also varied. While cNeuroD, cNeuroM, and cPhox2a expression remained relatively strong in the forming ganglia (Figs. 3Ci and 3Di), cNgn1 was only detected in cells in and immediately adjacent to the placodes (Figs. 3Bi–3Biii). An interesting difference to this pattern was seen in the forming geniculate ganglion where Phox2a expression was lost as cells formed the ganglion, but reappeared in the ganglion by day three of development (data not shown).

In order to make a more precise comparison of the relative timing of expression during this cellular process, we performed a series of double *in situ* hybridization experiments (Fig. 4). In brief, the general sequence of activation is: cSox3 > cNgn1 > cNeuroD > cNeuroM > cPhox2a. As placodally derived cells mature further, expression of these genes is lost. The timing of sequential loss of expression is cSox3 followed by cNgn1, while Phox2a remains expressed in the ganglion cells (except geniculate as discussed above) and cNeuroM and cNeuroD remain expressed in the ganglion cells as late as 8 days of development (Roztocil *et al.,* 1997). As in mice, this expression shows a sequential series of gene switching. However, the precise series of genes and expression periods differs significantly from mice, most notably the expression of cNgn1 rather than cNgn2, and the much earlier expression of several genes (Fode *et al.,* 1998; see Discussion). In order to verify this difference, we carried out comparison between Ngn1 and Ngn2 expression between chick and mouse. These results confirmed the previously reported expression in mice (Fode *et al.,* 1998; Ma *et al.,* 1998, 1999) where mNgn1 was most strongly expressed in the trigeminal region and mNgn2 was more prevalent in the epibranchial region, with lower levels of each gene in the alternative domain (data not shown). In chick, each gene was expressed in the opposite domain to that seen in mice; cNgn1 is expressed predominantly in the epibranchial region while cNgn2 was detected only in the trigeminal region.

A striking feature of the region of neurogenesis was that it was restricted to the dorsal part of the cSox3 domain (Figs. 4Aii–4Dii). However, at later stages (HH stage 21/22), expression of cNeuroD was seen throughout the cSox3-positive domain, although the region from which migration was apparent remained restricted to the dorsal part of the placode (data not shown).

In summary, the regions of neurogenesis from surface ectoderm lie within the regions of cSox3 expression. cSox3 transcripts are lost as cells leave the ectoderm and the sequential expression of transcription factors is seen, although the precise details differ significantly from that previously reported in the mouse.

Effects of cSox3 Overexpression

Migration. During the second and third days of development, many cells delaminate and migrate inwards from the placodes and concomitantly undergo neurogenesis. As shown above, these cells lose expression of cSox3 by the time they leave the ectoderm. We therefore set out to determine whether the loss of cSox3 was a necessary event for cells to delaminate. This was achieved by maintaining cSox3 expression in the ectoderm by electroporation of a GFP-cSox3 expression construct into the epibranchial region of embryos at HH stage 14 and analyzing the position of the transfected cells 24–48 h later at stage 18–20.

Control embryos transfected with vectors expressing either GFP alone or GFP fused to antisense orientation of cSox3 exhibited a high degree of inward migration (14/14). Many cells could clearly be seen populating the forming petrosal ganglia (Figs. 5G–5J). In contrast, when embryos were transfected with sense cSox3 constructs, the number of labeled cells migrating inwards was much reduced (Figs. 5B–5D). However, some cells staining for the presence of the ectopic cSox3 were sometimes seen beneath the ectoderm (10/18 embryos; Figs. 5C and 5D). Expression constructs were also introduced into other regions of ectoderm. Interestingly, when transfection of sense-cSox3 constructs was seen in the region of the trigeminal placode, the level of inward migration did not differ between controls and embryos transfected with a cSox3 expressing construct (8/8 embryos, Fig. 5A). In order to quantify this low level of migration in those embryos where migration was observed from the placodes, we counted cells migrating in the region of each placode (Fig. 6), as defined by the region of neurogenesis (staining positive for Hu). This analysis showed that the number of migrating cSox3-transfected cells was only 14–22% (geniculate), 4–10% (petrosal), 6–18% (nodose I) as compared to the number migrating from the trigeminal placode. These show a pronounced effect as compared to values of 87% (geniculate), 100% (petrosal), 66% (nodose I) in the GFP control. These effects of cSox3 transfection represent some of the least severe effects on migration, since many embryos, 8/18, showed a complete loss of migration from the epibranchial placodes as discussed above. The reason we see some cells migrating when apparently overexpressing cSox3 is not certain. One possible explanation is the lag period between transfection and expression of the protein produced by the transgene. GFP is first detectable only 3–4 h after electroporation and it seems likely that the cells we observed which had migrated despite the presence of the cSox3-expressing transgene may have done so before the protein was expressed. These cells may then halt their migration or be unaffected by the reappearance of the cSox3 protein.

Hence, it appears that overexpression of cSox3 interferes with inward migration from the epibranchial placodes, which normally express cSox3, but not from the trigeminal placode that does not normally express this gene.

Ectodermal morphology. In the above experiments, many cells outside of the placodes were also transfected.

We frequently observed (26/31) that the transfected cells on the surface adopted a different morphology to their non-transfected neighbors (Fig. 7). In all cases, some regions of transfected ectoderm adopted a thickened morphology reminiscent of the epibranchial placodes (Figs. 7A, 7B, 7G, and 7H). In four cases, structures with a thick concave shape reminiscent of the developing lens were also formed, all of which were between the otic and optic region of the head (Figs. 7C–7F). In three cases where ectoderm of the trunk was also transfected, we observed disorganized piling up of cells (Figs. 7I–7J). On no occasion did we see such structures in embryos transfected with control plasmids.

DISCUSSION

cSox3 and Epithelial Morphology

We have shown that development of the neurogenic epibranchial placodes of the chick is marked by expression of the cSox3 gene. This gene is also expressed in other ectodermal placode structures such as the olfactory, otic, and the non-neuronal lens placode. The only exception is the trigeminal placode, which, although often included in the placode family, is the only one that does not exhibit a general thickened epithelial morphology (Kamachi *et al.,* 1998 and our data, not presented). Given its expression in the lens and lack of expression in the trigeminal placode, we suggest that expression of cSox3 relates primarily to the thickened morphology of the placodal structures rather than neurogenic capacity. We have shown that cSox3 expression is lost as the epithelium thins outside of the placodal regions during normal development (see also Ishii *et al.,* 2001). When cells later migrate away from the epithelial placodes during normal neurogenesis, they also lose cSox3 expression. This loss of cSox3 expression in cells as they migrate is also true of the earliest expression of cSox3 in the epiblast at the time of gastrulation, when cells moving into the primitive streak lose cSox3 expression as they delaminate from the epithelial epiblast (Rex *et al.,* 1997b). Likewise, later expression of cSox3 in the CNS is restricted to the proliferative neuroepithelium and is lost from cells as they migrate out from it (Uwanogho *et al.,* 1995; Rex *et al.,* 1998).

In all of these situations, the correlation between loss of cSox3 expression and the change in epithelial state is maintained. Thus, expression of cSox3 might maintain cells in a thick epithelial state which is only lost when cSox3 expression ceases. Consistent with this, we found that overexpression of cSox3 often caused the ectoderm to adopt a thick epithelial morphology outside of the normal placodal domain. The fact that overexpression of cSox3 resulted in such morphological changes in the surface ectoderm supports an active role for cSox3 in controlling epithelial organization. However, these changes, resulting in a well organized thickened epithelium, were never seen in more posterior regions of the embryo trunk, suggesting that other factors are necessary for cSox3 to exert this effect. Interestingly, the morphology induced by cSox3

overexpression seems to reflect the location of the structure, with lens-like structures near the eye and epibranchial placode-like structures in the branchial region. This suggests that cSox3 may be involved in initiating placodal morphology with other aspects of placode identity being defined by more local signals.
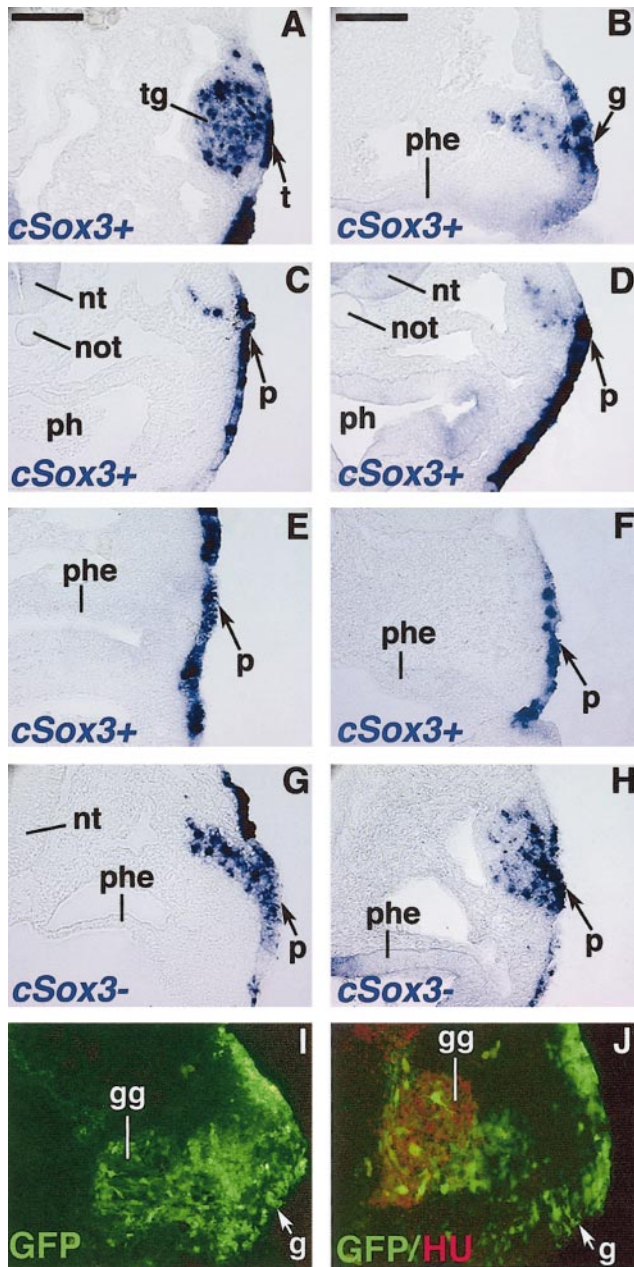
The piling up of cells, especially as seen in the trunk where the occasional structures were less organized, might relate to the recently published observation that cSox3 can act as an oncogene (Xia *et al.,* 2000). In this study, overexpression of cSox3 in chick embryo fibroblasts led to the appearance of transformed foci. However, overexpression of Sox3 in Medaka (Köster *et al.,* 2000), and generally in our system, did not lead to any pronounced increase in proliferation except the occasional example in the trunk. This suggests that the oncogenic potential of cSox3 can only be realized in somewhat alien situations. Elsewhere, the effects of cSox3 expression may be modulated by other factors involved in its normal function. It remains possible that longer term expression of cSox3 could result in tumorogenic growth, but we have yet to test this possibility.
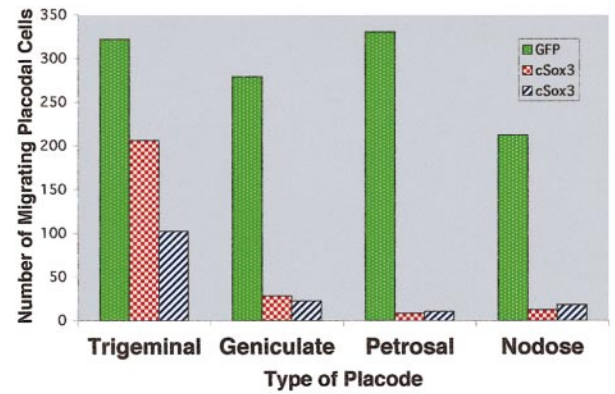
cSox3 and Competence

We have shown how expression of cSox3 has allowed us to follow the development of epibranchial placodes. This shows that the appearance of four discrete placodes is preceded by the presence of broader regions of ectoderm at earlier stages that also exhibit thick epithelial morphology. This gradual restriction of cSox3 expression is due to loss of expression of cSox3, and thinning of the ectoderm, outside of the placodal patches rather than migration of cSox3-positive cells (Ishii *et al.,* 2001). As discussed above, our data suggest that the loss of cSox3 expression might be a direct cause of the ectodermal thinning.

It has been suggested that the region of competence to respond to placode-inducing signals (in this case meaning signals which induce neurogenesis) is restricted to the regions of ectoderm which remain thick (Graham and Begbie, 2000). Thus, cSox3, which is expressed exclusively in the thickened ectoderm, would represent a marker of such competence. A recent study in the fish, Medaka, demonstrated that overexpression of Sox3 from a very early stage of development resulted in the appearance of ectopic lens and otic placodal structures (Köster *et al.,* 2000). These observations suggest that Sox3 expression may give ectoderm competence to respond to signals that induce the epithelial placodes themselves. The recent study of Begbie *et al.* (1999) using *in vitro* culture of ectoderm with or without pharyngeal endoderm clearly demonstrated the ability of endoderm to induce neurogenesis from surface ectoderm. The signaling peptide, BMP7, was shown to be necessary and sufficient to initiate this neurogenesis and is expressed in a manner consistent with it being the endoderm-derived signal. However, it is not clear whether there is an alternative signal giving rise to the cSox3-expressing placodes, which are then competent to respond to the neurogenic signal, BMP7, from the endoderm. Using

FIG. 6. Graph showing the number of migrating placodal cells in the trigeminal, geniculate, petrosal, and the first nodose placodes in a GFP-transfected embryo (illustrated as a green column) in comparison with two cSox3 transfected embryos (illustrated as red and blue columns, respectively). All embryos were transfected at HH stage 14, incubated, and analyzed at HH stage 18. The number of migrating placodal cells indicates the placodal cells that either delaminated and migrated from the ectodermal placodes or already reached the ganglia. The area within which migrating cells were counted was defined by staining for cells undergoing neurogenesis using the anti-Hu antibody.

cSox3 as a marker, this question can now be resolved using tissue transplantation and treatment with candidate signaling molecules.

Ectoderm outside of the presumptive placode regions has previously been shown to be capable of contributing neurons to the cranial ganglia until at least HH stage 12 in chicks (Vogel and Davies, 1993). This has been taken as evidence that it is capable of being induced to become placodal. However, in an *in vitro* co-culture system, ectoderm from the trunk was not responsive to endoderm, while ectoderm from the cranial regions that was not fated to become placodal was responsive (Begbie *et al.,* 1999). This difference may be due to a prior signal that is needed to "prime" the ectoderm to become placodal. Given the observation that ectopic expression of Sox3 in Medaka can result in ectopic placodal structures, it seems possible that Sox3 marks such primed ectoderm. Indeed, the region of ectoderm that was competent to respond to endoderm appears to correlate well with the cSox3-positive ectoderm at that stage, while the unresponsive ectoderm would generally be cSox3-negative. We can now address this issue in more detail. First, does the region of competence corre-

FIG. 5. Effect of cSox3 overexpression on inward migration of cells from placodes. Presence of ectopic plasmid was detected by *in situ* hybridization (A–H) or by GFP expression (I, J) 24–48 h after electroporation. Sections are at the level of the trigeminal (A), geniculate (B, I, J), and petrosal (C–H) placodes. (A) Ectopic cSox3 in the trigeminal placode demonstrating normal migration of labeled cells into the ganglion. Ectopic cSox3 in the geniculate (B) and in the petrosal (C, D) placodes, respectively, illustrating a low level of migration inwards (sections shown illustrate the greatest level of migration seen in these embryos). (E, F) Ectopic cSox3 in the petrosal placodes showing lack of migration of the labeled cells. (G, H) Anti-sense cSox3 controls showing cells undergoing normal inward migration into the ganglia from the petrosal placode. (I, J) GFP controls illustrating expression from the pEGFPC1 vector in the geniculate placode and co-stained for Hu in red (J). cSox3+,

sense construct; cSox3−, antisense construct; t, trigeminal placode; tg, trigeminal ganglion; g, geniculate placode; gg, geniculate ganglion; p, petrosal placode; nt, neural tube; not, notochord; ph, pharynx; phe, pharyngeal endoderm. Scale bar, approximately 100 μm.
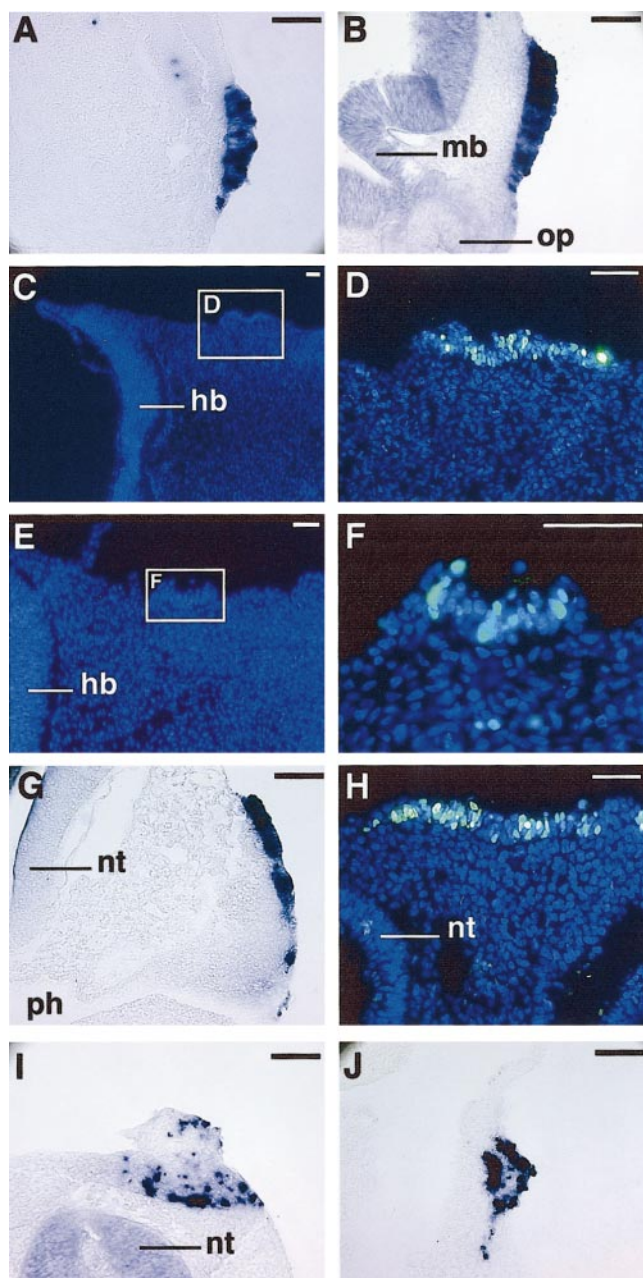
FIG. 7. Abnormal ectodermal morphology caused by ectopic cSox3. *In situ* hybridization for ectopic sense cSox3 plasmid stained in blue (A, B, G, I, J) or GFP-cSox3 fluorescence and DAPI staining (C–F, H) 24 h after electroporation (HH stage 18). Abnormal morphology of transfected ectodermal cells anterior to the otic vesicle (A–F), in the branchial region (G–I), and in the trunk (J). Note concave placode-like structures in some transfected domains anterior to otic vesicle (C–F) and disorganized piling up of cells in the head and trunk (I, J). mb, midbrain; hb, hind-brain; op, optic cup; nt, neural tube; ph, pharynx. Scale bar, approximately 100 μm.

spond to regions of cSox3 expression? Second, does induction of nonplacodal ectoderm result in activation of cSox3, or can neurogenesis take place independently of cSox3

expression? Our failure to detect cSox3 expression in the trigeminal placode suggests that cSox3 expression is unlikely to be an absolute requirement for neurogenesis.

Placodal Neurogenesis

We have shown the sequential expression of several bHLH genes as cells undergo neurogenesis from the epibranchial placodes. This identified a number of differences between mouse and chick. The first type of difference was the timing of expression with three genes, Ngn1, NeuroD, and NeuroM, being expressed prior to delamination in chick while their expression has been described only after delamination in the mouse. However, due to the limited data published from mouse, it is not possible to determine how genuine this difference is at present. The second difference concerns the expression domains for Ngn1 and Ngn2 in the epibranchial or trigeminal placodes, respectively, in chick, which is inverted in mouse. Such inversion is not unprecedented, with similar alternative distribution of transcription factors described in the developing brain (Sox4 and Sox11) (Cheung *et al.,* 2000). The significance of this difference between chick and mouse is not clear. It may be that either gene can carry out the necessary function in either region, although the presence of two genes suggests that the function of both genes must be necessary, providing some particular feature of either epibranchial or trigeminal neurons. Further functional experiments will be needed to determine whether the difference between chick and mouse is reflected in differences in the neurons produced.

The differences between gene expression in the epibranchial placodes as compared to the trigeminal placodes demonstrate a clear distinction between these two types of neurogenic placodes. The epibranchial placodes express cSox3 and then cNgn1 (Ngn2 in mouse), cPax2 and later cPhox2a, while the ophthalmic trigeminal placode does not express cSox3, expresses cNgn2 (Ngn1 in mouse), cPax3 rather than cPax2, and later does not express cPhox2a (Baker and Bronner-Fraser, 2000). It therefore seems that there is a high level of similarity shared between the four epibranchial placodes, which is not shared by the trigeminal placode.

Our experiments in which cSox3 was overexpressed and thus maintained in the placodal ectoderm demonstrated that migration from the placodes was disrupted by continued expression of cSox3. Together with the effect seen on epithelial morphology outside of the placodes, this suggests that cSox3 expression might be involved in maintaining cells in an intact epithelium and loss of expression is necessary to release cells from that epithelium. Interestingly, overexpression of cSox3 in the region of the trigeminal placode did not result in inhibition of inward migration in the transfected cells (Fig. 5A). This suggests that the effect of cSox3 overexpression seen in the epibranchial placodes is context-dependent and may require other cofactors that are not present in the trigeminal placode.

It is interesting that the region of active neurogenesis, as

marked by the expression of the various neurogenic transcription factors in migrating cells, is generally not at the closest point of apposition between the ectoderm and endoderm in the dorsoventral axis (see Figs. 3Aii and 4Cii). This region is, however, generally overlapping the dorsal edge of the cSox3 expression domain. A possible explanation for this location would be that cSox3 expression is necessary to provide some aspect of competence for neurogenesis to be induced, but, as we have shown, expression of cSox3 must also be lost for cells to undergo normal neurogenic development. Given the location of the cSox3 domains, it seems likely that cSox3 expression is also positively regulated by the endoderm. Hence, endoderm-derived signals would maintain cSox3 expression and then later activate neurogenesis. Thus, only in a region where the signal to activate neurogenesis, and therefore downregulate cSox3, overrode the signal to maintain cSox3 expression, would neurogenesis occur.

What Is a Placode?

Placodes have long been defined as thickened regions of the surface ectoderm. Expression of cSox3 definitively identifies those placodes that do fit such a definition. It is clear from our study that the region of neurogenesis, presumably induced by BMP7 from the endoderm, is within the thickened cSox3 domain but not throughout this domain. However, as discussed above, the region of ectoderm competent to undergo neurogenesis is likely to coincide with the domain of cSox3 expression, even at early stages. Thus, cSox3 expression might define the region of thickened ectoderm that can undergo neurogenesis throughout the development of the epibranchial region and therefore fits the definition of neurogenic placode well. Therefore, by this definition, the placode is not the thickened ectoderm that undergoes neurogenesis but the thickened ectoderm *capable* of undergoing neurogenesis.

Thus, placodes are made up of two overlapping groups: the thickened placodes including the non-neurogenic lens placode, and the neurogenic placodes including the non-thickened trigeminal placode. A looser definition incorporating all of these is currently in general use and seems the most expedient at present. However, when unraveling the cellular and molecular mechanisms underlying the development of the vertebrate nervous system, it is important to distinguish between events involved in development of the placodes and those regulating neurogenesis from the placodes. cSox3 now provides a valuable tool to investigate these processes.

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