Extensive Cell Movements Accompany Formation of the Otic Placode

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During development, the vertebrate inner ear arises from the otic placode, a thickened portion of the ectoderm next to the hindbrain. Here, the first detailed fate maps of this region in the chick embryo are presented. At head process stages, placode precursors are scattered throughout a large region of the embryonic ectoderm, where they intermingle with future neural, neural crest, epidermal, and other placode cells. Within the next few hours, dramatic cell movements shift the future otic placode cells toward the midline and ultimately result in convergence to their final position next to rhombomeres 5–6. Individual cells and small cell groups undergo constant cell rearrangements and appear to sort out from nonotic cells. While the major portion of the otic placode is derived from the nonneural ectoderm, the neural folds also contribute cells to the placode at least until the four-somite stage. Comparison of these fate maps with gene expression patterns at equivalent stages reveals molecular heterogeneity of otic precursor cells in terms of their expression of \( dlx5 \), \( msx1 \), \( Six4 \), and \( ERN1 \). Although \( Pax2 \) expression coincides with the region where otic precursors are found from stage 8, not all \( Pax2 \)-positive cells will ultimately contribute to the otic placode. © 2002 Elsevier Science (USA)

Key Words: chicken; ear development; fate map; placode field.

INTRODUCTION

In vertebrates, the inner ear is one of the most complex sensory organs comprising the semicircular canals, up to seven different sensory organs or patches, and the cochlea housing the auditory apparatus. Despite this complexity, the inner ear (along with other sensory organs and cranial ganglia) arises from simple ectodermal thickenings, the cranial placodes, which lie adjacent to the anterior neural plate (for review, see Anniko, 1983; Fritzsch et al., 1998; Torres and Giraldez, 1998). The otic placode first becomes morphologically discernible around the 10-somite stage, next to rhombomeres 5 and 6 of the hindbrain (Bancroft and Bellairs, 1977; Haddon and Lewis, 1996; Romanoff, 1960; Schlosser and Northcutt, 2000). It then invaginates (while neuroblasts delaminate to give rise to the cochlear vestibular ganglion) and eventually separates from the surface ectoderm. During subsequent morphogenetic events, the otic vesicle generates the complex architecture of the inner ear.

Both initial formation and later patterning of the otic placode are controlled by inductive interactions with surrounding tissues. For example, it has been proposed that the otic placode is induced by sequential signals emanating from different tissues at different times and that therefore cells become gradually committed to otic fate (Gallagher et al., 1996; Jacobson, 1963a–c; Waddington, 1937; Yntema, 1933, 1950). Indeed, signals from the mesoderm and the neural plate play an important role during otic induction (Gallagher et al., 1996; Jacobson, 1963a; Mendonsa and Riley, 1999; Orts-Llorca and Jimenez-Collado, 1971). In addition, recent studies have confirmed that at least two different signals—FGFs and Wnts—promote the formation of the otic placode (Ladher et al., 2000; Phillips et al., 2001; Vendrell et al., 2000). However, none of these signals was shown to initiate otic development in naive ectoderm, and therefore it is still unclear whether they control the first step in otic induction or whether there are more upstream signals involved. Therefore, the question of when otic induction begins still remains open.

Inductive interactions can be revealed by comparing specification maps with fate maps made at the same stages.

Supplementary data for this article are available on IDEAL (http://www.idealibrary.com).

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FIG. 1. Standardization of measurements. Dil- and DiO-labelled positions and gene expression domains were measured by using visible landmarks as described in the text. (A) In stage 5–7 embryos, the position along the anteroposterior axis was expressed as % of the distance between the center of Hensen's node (0%) and the tip of the prechordal plate (100%; hn-pp). The mediolateral position was calculated as % of the distance between the midline (0%) and the border of the area pellucida (100%; ml-ap). (B) In embryos with two or more somites, the distance between the center of Hensen's node (0%) and the anterior edge of the first somite (100%; hn-som) was measured, and the anteroposterior position of labelled cells was determined as % of hn-som. Mediolateral position is expressed as % of the width of the mediolateral extent of the neural plate (np = 100%). (C, D) Plots to determine the degree of shrinkage along the mediolateral axis suffered by embryos after in situ hybridization. The x-axis shows the position of labels as measured in the living embryo, and the y-axis shows the same labels after in situ hybridization. In both cases, the relative measuring system explained in (A) and (B) above was used. These (and similar plots for other stages) were used to compare the position of gene expression domains to the fate maps. (E) Five cell populations of different sizes were labelled with Dil in a stage 5 embryo. The dye was then photoconverted with DAB and the embryo processed for whole-mount in situ hybridisation with an ERNI antisense probe. (Inset) Section through the same embryo at the level of labelling.
any differences between the two are likely to indicate the regions and times at which tissue interactions are required for cells to become determined to specific fates. Recently, Groves and Bronner-Fraser (2000) have reported that chick ectoderm isolated from the otic region at or later than stage 8 retains Pax2 expression, but not BMP7, Sox3, or Notch (which are specified at stage 9–10). However, most fate maps produced to date that include the otic territory have concentrated on early stages of development (primitive streak stages), when otic precursors are distributed over a large area of the epiblast and appear to be intermingled with precursors for other tissues. No detailed maps are available between the primitive streak stage and the onset of expression of the first otic marker Pax2, which might allow us to determine at what stage otic precursors first become confined to a discrete domain.

Here, a detailed fate map of the chick otic placode between head process and four-somite stages is presented. Our data reveal that, even after primitive streak stages, otic precursors continue to be interspersed with future neural, neural crest, epidermal, and epibranchial placode cells. Although the otic domain gradually condenses, cells of different fates remain mixed within it even after the onset of Pax2 expression. Comparison of fate maps at different stages suggests that extensive cell movements accompany placodal development. This was confirmed by time-lapse microscopy, which revealed for the first time that scattered cells and cell groups converge to the placode while constantly undergoing cell rearrangements. Even at fairly late stages, cells arising from the ectoderm along most of the hindbrain and from the neural folds contribute to the placode. Finally, a detailed comparison is made between otic fate and the expression patterns of several genes at the border of the neural plate, including the prospective otic region. This comparison reveals that otic cells are recruited from a molecularly heterogeneous cell population, through extensive migration.

**MATERIALS AND METHODS**

**Embryo Techniques**

Fertile hen eggs (Winter Farm, Hertfordshire, UK) were incubated at 38°C for 20–44 h to obtain embryos at stages 5–10 (Hamburger and Hamilton, 1951). Stage 5 and 6 embryos were explanted in Pannett Compton saline and maintained in New culture (New, 1955) modified after Stern and Ireland (1981), while older embryos were cultured in ovo. For video time-lapse analysis,
embryos were cultured dorsal side up on agar-albumin plates (Chapman et al., 2001). For fate-mapping studies, small groups of epiblast cells were labelled by using the fluorescent dyes DiI and/or DiO as described previously (Stern, 1998). Briefly, stocks of 0.5% DiI or 0.25% DiO in absolute alcohol were diluted 1:10 in 0.3 M sucrose at 50°C and injected by air pressure using a micropipette made from 50-μl borosilicate glass capillaries. The labelled position was determined in relation to other landmarks (see below), and the embryos were then cultured until the otic placode or cup could be identified by morphological criteria (stage 12–15). The fate of the labelled cells was assessed in whole mounts, in 40-μm vibratome sections, or after photo-oxidation and wax sectioning. Photo-oxidation using 3,3'-diaminobenzidine (DAB; Sigma) was performed as described previously (Izpisua-Belmonte et al., 1993).

**Standardization of the Position of Labelled Cells**

The anteroposterior and mediolateral positions of DiI- and/or DiO-labelled cells were measured by using an eyepiece graticule immediately after injection. In stage 5–7 embryos, distances were determined from the centre of Hensen’s node (primitive pit) to the tip of the prechordal plate (hn-pp = 100%; see Fig. 1A) and to the labelled cells, respectively. The position of the label was calculated as a percentage of the total length hn-pp. To standardise the mediolateral position, the distance between the midline and the labelled cells was expressed as % of the distance between the midline and the edge of the area pellucida (ml-ap = 100%; see Fig. 1A).

In embryos with two somites or more, distances were measured from the primitive pit to the anterior edge of the first somite (hn-som = 100%; see Fig. 1B) and to the labelled cells. The position of the label was standardized as a percentage of the total distance hn-som. The mediolateral position of the labelled cells was expressed as a percentage of the distance between the midline and the lateral edge of the neural plate (np = 100%).

Note that, using this system, these measurements are relative such that the 100% value differs considerably between the mediolateral and anteroposterior axes, as well as between different stages. For example, at stage 5, the ml-ap distance (100%) averages 1012 ± 97 μm and the hn-pp distance (100%) corresponds to 1250 ± 46 μm. At the two-somite stage, the hn-som distance (100%) averages 434 ± 46 μm, while the np distance (100%) is 200 ± 20 μm.

**Video Time-Lapse Filming**

Four different positions of the epiblast on each side of stage 6–8 embryos were labelled with DiI (0.025% in 0.3 M sucrose; diluted 1:20 from a 0.5% stock solution in absolute alcohol). The embryos were cultured in a heated chamber placed around a Zeiss Axiolab with fluorescence optics. A transmitted light and a fluorescence image were captured with a 5× objective every 10 min by using a Princeton Instruments cooled CCD camera and Metamorph software at a resolution of 658 × 517 (24-bit color). The two images were overlaid by encoding the fluorescence image in the red channel and the transmitted light image in both the green and blue channels. The resulting time-lapse series was saved as a stack typically consisting of 150 color-encoded images. For posting as supplementary material, the stacks were converted to AVI files by using Cinepak (compressed by radius).

**Whole-Mount in Situ Hybridization and Histology**

cDNAs for dlx5 (Ferrari et al., 1995), msex1 (Liemb et al., 1995), Six4 (Esteve and Bovolenta, 1999), and Sox2 (Kamachi et al., 1995; Uwanogho et al., 1995) were kind gifts from R. A. Kosher, T. M. Jessell, P. Bovolenta, P. Scotting, and R. Lovell-Badge, respectively. ERNI has been described previously (Streit et al., 2000). Whole-mount in situ hybridization using DIG- and FITC-labelled anti-sense RNA-probes was performed as previously described (Stern, 1998; Streit et al., 1997; Théry and Stern, 1996). To detect a single transcript, embryos were developed by using NBT/BCIP as a substrate (Sigma), embedded in paraffin, and sectioned (10 μm). For double in situ hybridization, NBT/BCIP (Sigma) and int-BCIP (Roche) were used as substrates and 40-μm sections were cut with a vibratome (Leitz).

**Determination of Gene Expression Domains**

The mediolateral extent of the expression domains of dlx5, ERNI, msex1, and Six4 and the position of the Pax2 domain were determined after whole-mount in situ hybridization by using the same criteria as described for the fate map (see Figs. 1A and 1B). For each stage and transcript, 5–10 embryos were measured. However, embryos subjected to in situ hybridization undergo considerable shrinkage, particularly along the mediolateral axis (Figs. 1C and 1D). To compensate for this shrinkage so that direct comparisons could be made between fate map results and gene expression domains, a calibration curve was drawn: spots of DiI were placed on the epiblast of 32 embryos (stage 5–8), the position of each spot was measured, and the embryos were immediately fixed, after which the dye was photoconverted by using DAB. They were then processed for whole-mount in situ hybridization by using Six4, dlx5, ERNI, or Pax2 probes, and both the expression domains and the position of the label were measured (Fig. 1E). The positions of the DiI label before and after hybridization were used to construct graphs like those in Figs. 1C and 1D. In embryos with 2 somites or more, no significant difference was found between both sets of measurements. However, in younger embryos, there was significant shrinkage, which was greater in lateral regions than close to the midline (see Figs. 1C and 1D). These graphs were used to extrapolate the position of gene expression domains to their presumed positions before in situ hybridization and to relate them directly to the fate map data.

**RESULTS**

The otic placode forms in the ectoderm adjacent to the neural plate just anterior to the first somite and can first be identified morphologically around the 10- to 14-somite stage (Bancroft and Bellairs, 1977; Haddon and Lewis, 1996; Romanoff, 1960; Schlosser and Northcutt, 2000). In the primitive streak stage chick embryo, future otic cells are distributed over a fairly large region of the epiblast, apparently interspersed with precursors for other tissues (Garcia-Martinez et al., 1993; Schoenwolf and Sheard, 1990). To understand how cells from this broad area converge to form the otic placode, a fate map was constructed from head process to the 4-somite stage by injecting the fluorescent dyes DiI and DiO to label small cell populations of the chick epiblast. The position of labelled cells was analyzed...
after the otic placode or vesicle had formed (stage 11–15). In total, 398 embryos were analyzed, most of which had received one Dil and one DiO injection on each side of the midline. Figure 2 shows some examples of Dil-labelled embryos.

Origin of the Otic Placode

Otic precursors converge from a broad region of the ectoderm to form the placode. At the head process stage (stage 5), otic placode precursors are distributed over a large region of the epiblast lateral to Hensen’s node and are intermingled with future neural tube, neural crest, epi-branchial placode, and epidermis cells (Fig. 3A). They span about one-third of the distance between the midline and the lateral edge of the area pellucida (see Table 1; total width ml-ap, 1012 μm; width of otic territory, 324 μm) and extend both anteriorly and posteriorly from Hensen’s node (about 25% of the distance between the node and the prechordal plate in both directions; hn-pp, 1250 μm). By head-fold stages (stage 6/7; Fig. 3B), otic precursors are still intermingled with cells of other fates, but have moved, together with future neural and neural crest cells, towards the midline (see Table 1; width of otic territory, 276 μm). They are now located anterior to Hensen’s node and spread about 500 μm along the anteroposterior axis (about 30% of hn-pp). Precursors for the epi-branchial placodes are mixed with future otic cells but extend more into the lateral ectoderm (ml-ap position 30–60%, 295 μm).

While they have largely separated from hindbrain precursors at the two-somite stage (except at the edge of the neural plate; Fig. 3C), many future otic cells are found in close association with neural crest precursors at the border of the neural plate. The otic domain, however, extends further laterally into the ectoderm (to np position 175%, 170 μm lateral to the border of the neural plate) than the neural crest territory (to np position 125%, 57 μm from the border of the neural plate) and overlaps laterally with precursors for the epi-branchial placodes (np position 130%–220%, width 180 μm). The density of otic precursors close to the region where the placode will form has increased.

During subsequent stages (Figs. 3D and 3E), the relative position of the future otic placode remains fairly constant and extends from the level of the future anterior hindbrain to the level of the first somite. Otic precursors have not yet separated from future epi-branchial or neural crest cells. The neural folds continue to give rise to neural, neural crest, and otic cells; even at the four-somite stage, the inner neural folds contribute cells to the otic placode.

Otic precursors do not arise in register with rhombomeres 5 and 6. Once the otic cup becomes visible, it lies opposite rhombomeres 5 and 6 of the hindbrain. How early is this alignment fixed? To address this, the rostro-caudal position of cells contributing to various levels of the neural tube and neural crest was analyzed and compared with the position of otic precursors (Fig. 4). At early stages (stage 5–6), otic precursors are mixed with cells that will contribute to a large region of the neural tube and neural crest, extending from the posterior midbrain to the anterior spinal cord. After this, they begin to condense at the level of future rhombomeres 2–7. However, even at later stages (stage 8, 4 somites), some future otic cells are still found at the same level as cells that will contribute as far anteriorly as rhombomere 1 and as far posteriorly as the rostral spinal cord.

Regional subdivision of the otic placode. From around stage 14, several molecular markers become restricted to subdomains of the otic placode, such as Pax2 (Herbrand et al., 1998; Hidalgo-Sanchez et al., 2000; Nornes et al., 1990), Gbx2 (Hidalgo-Sanchez et al., 2000), and BMP7 (Groves and Bronner-Fraser, 2000) medially, and SOHo (Kiernan et al., 1997), Otx2 (Hidalgo-Sanchez et al., 2000; Morlali et al., 1999), Hmox3 (Herbrand et al., 1998), and GH6 (Kiernan et al., 1997) laterally (for review, see Brigande et al., 2000). These domains of gene expression correlate approximately with areas destined to form the cochlear (ventromedial) and vestibular (lateral) portions of the inner ear (see Li et al., 1978). Do these subdomains of the otic cup arise from separate groups of precursors? To address this, it was determined whether specific positions of the early fate maps give rise to cells restricted to specific quadrants of the otic vesicle (Fig. 5). Although there is a general tendency for more posteriorly located precursors to contribute to the posterior portion of the otic vesicle, and similarly for the mediolateral axis, there is no clear subdivision of the fate map into individual compartments at any of the stages investigated. Even at later (4-somite) stages, cell groups that will contribute progeny to opposite poles of the otic vesicle are often found adjacent to each other. These findings are in agreement with a recent study in Xenopus, showing that cells continue to mix extensively even after formation of the otic vesicle (Kil and Collazo, 2001).

In summary, our results indicate that the otic placode arises from a very broad region of the embryonic epiblast. Up to the one-somite stage, its precursors are intermingled with cells that contribute to the epidermis, the epi-branchial placodes, the central nervous system, and neural crest cells at all levels between the anterior and posterior hindbrain. There is no early regional subdivision of the presumptive otic territory. Otic precursors accumulate at the border of the neural plate and even at fairly late stages cells from the inner neural folds are recruited into the placode. Comparison of the position of otic precursors at different stages suggests that extensive cell movements accompany the formation of the otic placode.

Extensive Cell Movements Lead to the Formation of the Otic Placode

The results presented above led to the possibility that formation of the otic placode is accompanied by massive cell movements. To confirm this and to visualize these movements directly, we used time-lapse video analysis. Three to four cell groups on each side of the embryo (stage
FIG. 3. Fate map of the otic placode between head process and 4-somite stages. Small cell groups in the epiblast were labelled with DiI and DiO and their fate determined after the otic placode or vesicle could be identified by morphology. (A) At stage 5, otic precursors are distributed among neural, neural crest, epidermal, and epibranchial placode precursors in a large region of the epiblast. (B) By stage 6–7, they have converged towards the midline to become located in the ectoderm, first anterior to Hensen’s node (stage 6), and later anterior to the first somite (stage 7). (C) By stage 7, the prospective otic territory has almost completely separated from future neural plate cells (except at the neural folds), but still contains precursors for other tissues. (D, E) During the 3- (D) and 4-somite (E) stages, the neural folds still contribute cells to the otic placode; otic precursors are also found in the epidermis adjacent to most of the hindbrain. The circles represent labels that gave rise to cells in a single location, while squares represent those where progeny was found in more than one tissue. Crossed circles represent positions labelled in the filmed embryo shown in Fig. 6 (see Supplementary Material). The dotted line in (C–E) indicates the visible edge of the neural plate. The grey oval in (D) and (E) approximately encircles the Pax2 expression domain, and the horizontal grey line shows the posterior boundary of Pax2 in the CNS. The mediolateral extents of the expression domains of msx1, dlx5, Six4, and ERNI are indicated by horizontal bars.
FIG. 4. Otic precursors are not aligned with R5 and R6 precursors. Diagrams showing the position of future otic cells (black circles) at stages 5–8 (A, stage 5; B, stage 6/7; C, stage 7; D, stage 8; E, stage 8), in relation to precursors for the neural tube (circles) and neural crest (squares) at different positions along the rostrocaudal axis. R1–R7, rhombomeres 1–7. The origin of neural crest cells was scored according to their final position based on data available in the literature (Noden, 1975; Lumsden and Keynes, 1989; Birgbauer et al., 1995); numbers in brackets reflect minor contribution of crest from these rhombomeres. Otic precursors arise from a very large rostrocaudal territory spanning the entire length of the hindbrain. The grey oval in (D) and (E) approximately encircles the Pax2 expression domain, and the horizontal grey line corresponds to the posterior limit of Pax2 in the CNS. The dotted line in (C–E) marks the position of the neural fold.

FIG. 5. Regionalisation of the otic vesicle. Diagrams showing the position of precursors for anterior, posterior, medial, and lateral otic placode at stages 5–8 (A, stage 5; B, stage 6/7; C, stage 7; D, stage 8; E, stage 8). The circles indicate labels that contributed cells to an entire half of the otic vesicle; the squares mark those that contributed to less than half (e.g., anterior–median quadrant). In no case did any label contribute to more than half of the otic cup. Note that cells contributing to different portions of the otic vesicle are extensively intermixed, even as late as stage 8 (4 somites).
6–8) were labelled with Dil to follow their movements over a 15- to 24-h period. Figure 6 shows frames from one representative movie (a compressed version of which can be seen in Supplementary material: http://www...). In agreement with the fate map data (see Fig. 3), of the four cell groups labelled on the left side, three contributed to the otic placode; while on the right, only progeny from the most medial cell group does. The diagram in Fig. 7A illustrates the movements of these four cell groups at 3-h intervals. The most dramatic movements are observed within the first 6–9 h, when lateral cells rapidly converge to the midline (see groups 1, 2, and 4 in Fig. 7A; see also Fig. 7C, groups 1 and 2). Afterwards, medially directed movements continue, but at a much slower rate. For example, by 9 h (Fig. 7A; and inset in Fig. 7A, turquoise outline), group 1 (mediolateral start position 41%) has fused with group 2 (mediolateral start position 28%) and during the next 6 h there is little directional movement; however, smaller groups of cells constantly change neighbors and eventually split off the main group to give rise to epidermis and the otic placode.

Cells from position 3 (Fig. 7A—shown in more detail in Fig. 7B) initially move as a coherent group towards the midline. After 12 h, the cells move away from the midline and split up into several groups (14 h). Figure 7B illustrates the movements of one such group over the next 360 min: after splitting off, the cells stall for about 100 min before they slowly begin to migrate away from the midline (during 180 min) and then rapidly (within 100 min) join the otic vesicle.

These results confirm that otic precursors converge to their final position from a large region of the embryonic ectoderm. During this process, extensive cell mixing occurs and some cell clusters derived from a coherent group of labelled cells split into smaller groups. Of these, some will contribute to the otic placode, while others will give rise to different tissues.

### Table 1

Comparison between the Position of Otic Precursors and Gene Expression Domains

<table>
<thead>
<tr>
<th>Stage</th>
<th>Medial border</th>
<th>Lateral border</th>
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<tbody>
<tr>
<td></td>
<td>msx1</td>
<td>dlx5</td>
</tr>
<tr>
<td>Stage 5</td>
<td>384 ± 14.4</td>
<td>404 ± 15.2</td>
</tr>
<tr>
<td>Stage 6/7</td>
<td>276 ± 18.7</td>
<td>342 ± 14.4</td>
</tr>
<tr>
<td>2 somites</td>
<td>190 ± 4.0</td>
<td>200 ± 6.0</td>
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Note. The mediolateral position of the expression domains of msx1, dlx5, Six4, and ERNI were measured at stages 5, 6/7, and 7 in 7–10 embryos of each stage. The average distance between the midline and the medial and lateral edges, respectively, of the expression domains is given in μm ± standard deviation (ml-ap in Fig. 1). The most medial and most lateral position in which otic precursors were found at different stages is also given in μm. At stage 5, none of the expression domains matches the mediolateral limits of the otic territory; from stage 6/7 onwards the medial border of the otic region closely correlates with the medial limit of msx1 expression, while its lateral border coincides with the lateral limit of Six4 expression.

### Relationship between Genetic Markers of the Border of the Neural Plate and the Future Otic Region

At head process stages, several genes, including dlx5 (Pera et al., 1999), msx1 (Streit and Stern, 1999), Six4 (Esteve and Bovolenta, 1999), and ERNI (Streit et al., 2000), are expressed at the anterior lateral border of the neural plate, a region that is thought to give rise to sensory placodes and cranial neural crest (for review, see Baker and Bronner-Fraser, 2001). To investigate which of these genes, if any, best reflects the mediolateral position of the future otic placode in the newly generated fate maps, their expression between stages 5 and 8 was compared to the fate map of the otic region by using single and double whole-mount in situ hybridization followed by histological sections.

Five to ten embryos of each stage were processed for in situ hybridization for each gene, and the domains of expression were measured by using the same criteria that were employed for the fate map (but correcting for shrinkage as explained in Materials and Methods; see Fig. 1). The results are summarized in Table 1 and Figs. 8 and 9.

At stage 5, dlx5, Six4, and ERNI transcripts are detected in a horseshoe shape surrounding the anterior neural plate with a caudal limit just posterior to, or at the level of, Hensen's node (Fig. 8A, a, e, and i). In contrast, msx1 expression is broad posteriorly, extending anteriorly along the border of the neural plate with a rostral limit just anterior to the node (Fig. 8A, m). During stages 6–7, dlx5, Six4, and ERNI domains maintain their posterior boundary relative to the regressing node (Fig. 8A, b, c, f, g, j, and k; Fig. 8B, a, b, f, g, p, and q), while msx1 expression moves anteriorly to surround the neural plate (Fig. 8A, n and o; Fig. 8B, k and l). Beginning at the two-somite stage, both ERNI and dlx5 are down-regulated in a posterior-to-anterior direction (Fig. 8A, d and h; Fig. 8B, c–e and h–j) and ERNI transcripts disappear completely by stage 9, while dlx5 becomes concentrated in the most anterior neural folds and the adjacent ectoderm (Fig. 8A, h). Six4 and msx1 remain...
expressed at the border of the neural plate (Fig. 8A, l and p; Fig. 8B, m–o, r, s). Medially, all four genes overlap with the expression domains of the neural markers Sox2 (Fig. 8A, q–t; Fig. 8B, u–y) and Sox3 (not shown) until stage 7/8, when only msx1 coincides with Sox2 in the inner neural folds. Thus, the expression of all four genes begins to overlap at node levels and subsequently spreads to encircle the entire anterior neural plate.

These expression domains appear very similar; to establish whether they are identical, we performed double in situ hybridization with various combinations of these markers (Fig. 9). The msx1 territory is narrowest and lies most medial, followed laterally by dlx5, Six4, and ERNI, all of which overlap with msx1 to different extents. Throughout all stages, this mediolateral sequence of expression is maintained.

Since the expression domains of these genes are not quite identical to each other, which, if any, correlates with the otic territory established in the fate maps? At stage 5, none of the gene expression domains can be related to the position of the otic precursors (see Table 1; Fig. 3A). However, like the otic territory, the msx1-, dlx5-, Six4-, and ERNI-positive regions shift towards the midline between stages 5 and 6 (Fig. 8A, b, c, f, and g), while Sox2 expression narrows (Fig. 8A, r). From stage 6/7, the mediolateral location of future otic cells corresponds approximately to the expression domain of Six4 except most medial (where Six4 does not overlap with msx1), while both ERNI and dlx5 are down-regulated from the otic territory after stage 7. Although some cells from the msx1 domain contribute to the otic placode, this domain never encompasses all otic precursors. In conclusion, therefore, the expression domains of genes that mark the border of the neural plate are not quite identical, and none of them relates directly to the position of precursors of the otic vesicle at stages 5–8 along either the mediolateral or the anteroposterior axis.

**Correlation of Pax2 Expression and Otic Fate**

Shortly after dlx5 and ERNI are downregulated (stage 7+), the transcription factor Pax2 begins to be expressed, first as a salt-and-pepper pattern within a domain that corresponds approximately to the otic region of our fate maps (cf. Figs. 10A–10D and 10L with Fig. 3). Subsequently, more cells within this domain begin to express Pax2 (Fig. 10E) until expression becomes uniform at stage 10 (Figs. 10F–10I). Do cells from the entire Pax2 domain contribute to the otic placode? To predict the Pax2 domain reliably, whole-mount in situ hybridization was performed on embryos at stages 8–10 (5–7 embryos at each stage), and the domain was measured with respect to other landmarks (see Fig. 1A). Using these measurements, we then labelled 3–4 small cell groups at different anteroposterior and mediolateral levels within the Pax2 domain with DiI and DiO (n = 17; Figs. 10J, 10K, and 10M–10S). To confirm that the labelled cells were indeed contained within the Pax2-positive region, 7 embryos (38 labels total) were fixed immediately after labelling, photo-oxidized, and processed for Pax2 in situ hybridization (Figs. 10J and 10K). Of these, 34/38 labels (90%) were found within the Pax2 domain. In another 10 embryos, the fate of cells labelled as above was assessed after the otic cup had formed. Most (9/10; 90%) showed DiO- and DiI-labelled cell groups in each otic vesicle (Figs. 10M–10S). Often, red and green cells were found in overlapping regions, confirming that, even at these fairly late stages (stages 8–10), cell mixing occurs frequently among cells that will contribute to the placode. However, some of the cells labelled within the Pax2 domain contribute to the epidermis, including the future epibranchial placodes (Fig. 10N; see also Fig. 3E). Together, these results suggest that, although cells derived from the entire Pax2-positive region can contribute to the otic placode, not all of them do.

**DISCUSSION**

**Dual Origin of the Otic Placode: Contribution of Neural and Nonneural Ectoderm**

In this study, a detailed analysis of the cellular movements that accompany the formation of the otic placode in the chick embryo is presented. Evidence is provided that cells fated to give rise to the otic placode originate from a large region of the epiblast at head process stages and converge towards their final position whilst they separate from other cell populations. In agreement with previous fate maps of primitive streak stage embryos (Garcia-Martinez et al., 1993; Schoenwolf and Sheard, 1990), this study shows that, at head process stages, otic precursors are intermingled with prospective neural tube, neural crest, epidermis, and epibranchial placodes. There is no clearly defined domain within which all cells are fated to become otic, as suggested by Rudnick (1944) based on her transection and explantation experiments. By stage 6/7, otic precursors are largely separated from future neural tube cells, but they continue to be mixed with prospective crest, epidermal, and epibranchial cells. Based on morphological criteria, it has been suggested that a proportion of otic cells is recruited from the neural plate and that, even at late stages of development, individual cells leave the neural plate to contribute to the placode (Mayordomo et al., 1998). Our results show that, while the major portion of the otic placode is derived from lateral ectoderm, a minor portion is indeed recruited from the inner portion of the neural folds (coexpressing msx1 and the neural marker Sox2) at least up to the four-somite stage, suggesting that the neural folds contain either multipotent precursors or mixed populations of cells with different fates. Indeed, it has previously been reported that single cells (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Selleck and Bronner-Fraser, 1995) in the caudal neural folds can give rise to neural tube, neural crest, and epidermal progeny. Likewise, small cell groups well within the caudal neural plate of embryos at stage 6–7 can contribute to both neural tube and epidermis (Brown and Storey, 2000). The present results reveal that
the otic placode has a dual origin: it arises from both neural and nonneural portions of the ectoderm.

**Regional Subdivision of the Otic Placode**

In fish, the prospective otic territory seems to be subdivided into anterior, posterior, medial, and lateral domains at midgastrula stages (50% epiboly; Kozlowski et al., 1997), whilst a recent study in Xenopus showed that extensive cell movements occur between different quadrants of the otic vesicle even after its closure (Kil and Collazo, 2001). The data presented here are more consistent with the results from the amphibian embryo, showing that no such subdivision can be demonstrated before stage 8 in the chick. The first molecular signs of regionalization of the chick otic placode appear around stage 14/15 (for review, see Brigande et al., 2000; Fekete, 1996), when, for example, Pax2 (Herbrand et al., 1998; Hidalgo-Sanchez et al., 2000; Nornes et al., 1990), Gbx2 (Hidalgo-Sanchez et al., 2000), and BMP7 (Groves and Bronner-Fraser, 2000) begin to be restricted to the medial placode, while SOHo (Kiernan et al., 1997), Otx2 (Hidalgo-Sanchez et al., 2000; Morsli et al., 1999), Hmx3 (previously Nkx5.1; Herbrand et al., 1998), and GH6 (Kiernan et al., 1997) are expressed in its lateral portion. Rota-
tion experiments revealed that, while the anteroposterior axis of the otic vesicle is set around stage 12, determination of the mediolateral axis begins shortly after asymmetric gene expression is observed but is not completed until much later (after embryonic day 3.5; Hutson et al., 1999; Wu et al., 1998).

**Coordinated Development of Neural and Placodal Structures?**

During sensory organ development, peripheral structures often arise in close association with the targets to which they later project, e.g., the future olfactory placode initially abuts the future olfactory bulb region before they become separated from each other by morphogenetic movements (Couly and Le Douarin, 1985, 1987, 1988; Whitlock and Westerfield, 2000). Neuroblasts delaminating from the otic placode give rise to the cochlear–vestibular ganglion, whose axons project to the auditory and vestibular nuclei of the brain stem. During development, these nuclei arise from rhombomeres 4–7 (Cramer et al., 2000; Marin and Puelles, 1995; Tan and Le Douarin, 1991). Otic precursors, however, are found in a much broader region of the ectoderm, from the posterior midbrain to the rostral spinal cord, indicating that they begin to align with the future auditory and vestibular nuclei only around the time that the placode becomes morphologically distinct. Neuroblasts migrating from particular rhombomeres follow unique routes and have defined fates (Chan and Tam, 1988; Lumsden and Keynes, 1989; Lumsden et al., 1991; Noden, 1975). Likewise, it has been suggested that cranial ectoderm may be subdivided into segments ("ectomeres"), aligned with the rhombomeres of the neighbouring hindbrain, and coordinated with specific neural crest migration routes (Couly and Le Douarin, 1990; Noden, 1993). This hypothesis predicts that boundaries exist that prevent cells in each ectomere from crossing into neighbouring ectomeres. The broad distribution of otic precursors along the entire hindbrain found in the present study does not support this idea. As late as the six- to eight-somite stage, otic precursors converge from different rhombomere levels to reach their final position adjacent to rhombomeres 5 and 6, suggesting that the ectoderm is not subdivided into lineally separate domains at this stage.

**A Common Placodal Field?**

It has been suggested (Jacobson, 1963c, 1966; for review, see Torres and Giraldez, 1998; Baker and Bronner-Fraser, 2001; but, Graham and Begbie, 2000; Schlosser and Northcutt, 2000) that all cranial placodes are derived from a single, common region—the "placodal field"—and that their initial induction involves a common molecular mechanism. In fact, a continuous ectodermal thickening

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**FIG. 7.** Movements of selected cell groups from filmed embryos. (A) Traces of the contours of four groups of labelled cells (numbered 1–4) from the filmed embryo shown in Fig. 6 (also shown in Supplementary Material), at 3- to 6-h intervals. Note that the most rapid movements occur in lateral regions during the first 9 h (e.g., group 1 was labelled at position 41% ml-ap; after 9 h [turquoise] it has fused with group 2, which started at ml-ap position 28%, see inset). Red, 0 h; blue, 3 h; turquoise, 9 h; purple, 12 h; yellow, 18 h; orange, 21 h. (B) In the same film, a small group of cells (white) has separated from group 3 at 14 h (grey outline); thereafter, it hardly moves for the first 1.5 h, then it slowly starts to move away from the midline for about 3 h, and finally moves rapidly towards the otic placode in the final 1.5 h of this sequence. The outlines of this cell group are overlaid onto the final frame of the film, dotted line showing the position of the otic cup. Grey, 0 min; light orange, 20 min; dark green, 80 min; pink, 120 min; blue, 140 min; purple, 160 min; yellow, 220 min; red, 240 min; light green, 260 min; dark orange, 280 min; brown, 300 min; turquoise, 320 min; white, 340 min; black, 360 min. (C) Movements of three cell groups in a different filmed embryo, which had been labelled at stage 6. Two groups (1, 3) contribute to the otic vesicles, while the third does not (2). Red, 0 h; blue, 3 h; turquoise, 9 h; purple, 12 h; brown, 15 h. Dotted lines in (A) and (C) demarcate the midline and the position of the otic cup at the final stage.
surrounding the anterior neural plate from which placodes arise has been observed in mouse (Verwoerd and van Oostrum, 1979), fish (Miyake et al., 1997), and amphibians (Knouff, 1935; Platt, 1896). The molecular network of Pax, Six, eya, and dac genes plays an important role in eye development, and members of these families are also expressed in other sensory structures, like the inner ear, the olfactory placode, and the cranial ganglia (for review, see Wawersik and Maas, 2000). The preplacodal region expresses Six1 and -4 (Esteve and Bovolenta, 1999; Ghanbari et al., 2001; Kobayashi et al., 2000; Pandur and Moody, 2000) and eya1 (Mishima and Tomarev, 1998; Sahly et al., 1999), while dach1 is expressed in the neural plate as well as in part of the placodal field (unpublished observations). In contrast, none of the Pax genes has been found in this region; instead their expression begins only in regions where placodes ultimately form, like the otic region (Groves and Bronner-Fraser, 2000; Hidalgo-Sanchez et al., 2000; Nornes et al., 1990; Pfeffer et al., 1998), Pax6 in the lens and olfactory (Hirsch and Harris, 1997; Li et al., 1994; Walther and Gruss, 1991), and Pax3 in the trigeminal placode (Baker et al., 1999; Stark et al., 1997). This raises the possibility that a common placodal region is set up by assembling part of the molecular network (Six, eya, and dach)—a molecular mechanism common to all placodes—and is then further refined by more localized signals that initiate Pax gene expression to establish placode identity.

A number of genes, among them dlx5 (Pera et al., 1999), msx1 (Streit and Stern, 1999), Six4 (Esteve and Bovolenta, 1999), and ERNI (Streit et al., 2000) are all coexpressed at the lateral border of the anterior neural plate and have variously been referred to as markers for the border, the neural crest (msx1), or placodal (Six4) region or the nonneural ectoderm (dlx5). Is the expression domain of any of these consistent with the idea of a common placodal field? Here, it is shown that the domains of expression of these genes are not identical, that they change over time, and that none of the genes ever encompasses the entire otic territory. All four transcripts overlap, but only partially, in a large portion of the ectoderm containing cells destined for many different structures. When otic precursors begin to accumulate at their final position anterior to the first somite, dlx5 and ERNI are down-regulated from this region, just a few hours before the first Pax2-positive cells appear and otic cells begin to be specified (Groves and Bronner-Fraser, 2000). Therefore, if a common placodal territory exists, inductive interactions or other mechanisms must exist to direct specific subgroups of cells to specific placodal fates from within this territory.

### Correlation between Pax2 Expression and Otic Fate and Possible Mechanisms of Placode Specification

The transcription factor Pax2 is generally regarded as an early otic-specific marker, although a direct comparison of its expression domain with regions containing future otic cells has never been made. Otic placode specification is thought to begin around the time when Pax2 is first expressed (Groves and Bronner-Fraser, 2000). Here, it is shown that, just like otic precursors, the earliest Pax2-positive cells to appear at stage 8 are scattered in a salt-and-pepper pattern within the region containing most, if not all, otic vesicles (see Fig. 10L). However, this territory also contains cells that do not contribute to this structure. It is conceivable that these early Pax2-positive cells correspond to cells specified to become otic. If so, the extensive cell movements described in this study could reflect an active process of cell sorting. Unfortunately, techniques to determine this directly are not yet available, particularly because shortly afterwards, Pax2 expression within this domain becomes uniform, which precludes the use of the Pax2 promoter to lineage-label the earliest expressing cells.

At the five- to eight-somite stage, otic precursors still arise from the entire Pax2 domain; however, nonotic cells also continue to originate from this territory. They subsequently move to the epiplacodal placodes, where they maintain or re-express Pax2, or they become epidermis and down-regulate expression. Thus, although the Pax2 expression domain correlates very well with the position of the prospective otic territory, not all cells from this domain will contribute to the otic region.

The findings presented here leave two main possible mechanisms by which otic placode specification could occur. Either individual cells are specified at an early stage...
(just before stage 8) through a widespread otic-inducing signal to which only some cells respond; this response is accompanied by the initial expression of Pax2 and is followed by the sorting out of these cells from their neighbours and migration to the site of the otic vesicle. Alternatively, otic-inducing signals are localized, cell movements in the epiblast occur randomly, and only those cells that reach the vicinity of the signal will be specified as otic. To distinguish between these possibilities, it will be necessary both to unravel the molecular cascade leading to otic induction as well as a molecular dissection of the regulatory elements controlling Pax2 expression.

By the four- to five-somite stage, fate (this study) and specification maps (Groves and Bronner-Fraser, 2000) for the otic placode overlap. At the same stage, FGF-19 and Wnt-8c, two signals that have been implicated in otic
induction (Ladher et al., 2000), become localized in the mesoderm underlyong and the neural tube adjacent to the future otic placode. Together, these observations suggest that the latter possibility is more likely and that otic induction is a late event. On the other hand, based on the present fate maps, all of the test tissues used in this study are likely to contain at least some cells with an otic fate, and the possibility therefore remains that FGF and Wnt are only permissive, rather than instructive. It will now be important to test whether these signals can induce otic fate and expression of otic-specific genes in regions that do not contain these cells.

FIG. 10. Pax2 expression demarcates the position of future otic cells. (A–D) Pax2 begins to be expressed in the otic territory in a salt-and-pepper pattern at stage 8. (E) The number of Pax2-expressing cells increases during stage 9 and the domain enlarges. (F–I) At stage 10, the otic expression of Pax2 is uniform. (J, K) DiI labelling of several cell groups (arrow heads: 6 groups in J, 8 in K) within the Pax2 domain: to ensure that the Pax2 domain could be labelled reliably, embryos were fixed immediately after labelling and the positions of the labelled cells measured. The dye was then photo-oxidized (brown; arrowheads) and the embryos processed for in situ hybridization with Pax2 (purple). All cell groups labelled are within the Pax2 domain. (L) Pax2 expression overlaps with the otic territory at stage 8. The dotted line shows the outline of Pax2 expression; the purple circles correspond to the labelling positions in Fig. 3E that contributed to the otic placode. (M–S) Three to four cell groups (numbered 1–7) in the Pax2 domain on each side of the embryo were labelled with DiI (red) or DiO (green). (M, O, R) Embryos immediately after labelling. (N, P, Q, S) Embryos after 21 h. (P, Q) The left and right otic cups, respectively, of embryo (O). Note that most of the cell groups (the only exception being groups 1 and 4 in M and N) labelled within the Pax2 domain contributed to the otic cup, but that extensive cell mixing has occurred between groups (e.g., arrows in S, where cells of group 2 have impinged into and split group 3). Some cell groups contributed to both the otic placode (arrow heads in N) and the adjacent epidermis (groups 2, 5, and 7 in M and N; group 1 in O and P; and groups 1 and 3 in R and S).
Cell Movements and Their Coordination

The results presented here reveal a very considerable amount of cell mixing and cell movement accompanying formation of the otic placode in the chick. In contrast, the otic placode in zebrafish (Kozlowski et al., 1997) and in amphibians (Carpenter, 1937; Röhl, 1983; Schlosser and Northcutt, 2000) is already confined to a fairly small region by the neurula stage or shortly thereafter and is well separated from precursors of the neural plate and other placodes. On the other hand, cell movements comparable to those described here have been observed for olfactory placode precursors in zebrafish (Whitlock and Westerfield, 2000). Here, olfactory cells arise from a broad region of the outer rim of the neural plate and converge to their final position adjacent to the future olfactory bulb. However, since in zebrafish otic-specific markers like Pax8, Pax2, and dlx3 (Akimenko et al., 1994; Ekker et al., 1992; Krauss et al., 1991; Pfeffer et al., 1998) are expressed much earlier than in the chick, it is possible that by neurula stages cell movements that direct otic cells into the placode region have already finished. Taken together, these findings could indicate that extensive cell movements are a characteristic of the formation of various placodes in different species, but may occur at slightly different developmental stages, raising again the possibility that cell sorting might be a general and conserved feature of placodal development. In the future, we will need not only to test the cell sorting hypothesis directly but also to elucidate the guidance mechanisms that direct future placodal cells to their final destinations. In this context, it is interesting to note that both the FGF and Wnt pathways have been implicated in the regulation of cell movements in a variety of systems (Whangbo and Kenyon, 1999; Heisenberg et al., 2000; for review, see Boilly et al., 2000; Montell, 1999; Wilson and Leptin, 2000).

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