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## Two regulatory genes, *cNkx5-1* and *cPax2*, show different responses to local signals during otic placode and vesicle formation in the chick embryo

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### SUMMARY

The early stages of otic placode development depend on signals from neighbouring tissues including the hindbrain. The identity of these signals and of the responding placodal genes, however, is not known. We have identified a chick homeobox gene *cNkx5-1*, which is expressed in the otic placode beginning at stage 10 and exhibits a dynamic expression pattern during formation and further differentiation of the otic vesicle. In a series of heterotopic transplantation experiments, we demonstrate that *cNkx5-1* can be activated in ectopic positions. However, significant differences in otic development and *cNkx5-1* gene activity were observed when placodes were transplanted into the more rostral positions within the head mesenchyme or into the wing buds of older hosts. These results indicate that only the rostral tissues were able to induce and/or maintain

ear development. Ectopically induced *cNkx5-1* expression always reproduced the endogenous pattern within the lateral wall of the otocyst that is destined to form vestibular structures. In contrast, *cPax2* which is expressed in the medial wall of the early otic vesicle later forming the cochlea never resumed its correct expression pattern after transplantation. Our experiments illustrate that only some aspects of gene expression and presumably pattern formation during inner ear development can be established and maintained ectopically. In particular, the dorsal vestibular structures seem to be programmed earlier and differently from the ventral cochlear part.

Key words: Homeobox, *Nkx5-1* gene, *Pax2*, Inner ear, Otic induction, Chick

### INTRODUCTION

The first morphologically discernible structures of inner ear development in the chick embryo are the otic placodes, which arise as two ectodermal thickenings lateral to the prospective rhombomeres 5 and 6 at the 3- to 5-somite stage. The placodes subsequently invaginate to form the otic pit at about the 12- to 14-somite stage and eventually the closed otic vesicles at the 24- to 30-somite stage (Knowlton, 1967; Anniko and Wikström, 1984). Transplantation experiments in amphibia and chick have demonstrated that the hindbrain plays a critical role in the development of the inner ear (Waddington, 1937; Yntema, 1955). It has also been suggested that the mesoderm underlying the auditory field may be important, however, it is unclear whether this influence is direct or mediated via neural induction (review: Van de Water and Represa, 1991). Several genes have been identified which are expressed during early ear development in the mouse, although their functions are largely unknown (review: Fekete, 1996). Gene inactivations generated either by homologous recombination, by chemical mutagenesis or by spontaneous mutations suggested that some genes expressed in the neighbouring hindbrain are involved in the molecular control of otic development. For instance, mice

carrying mutations in the *FGF3*, *Hoxa-1* or *kreisler* genes exhibit disrupted development of the hindbrain and malformations of the inner ear in line with the general concept of inductive interactions between hindbrain and otic placode (Mansour et al., 1993; Mark et al., 1993; Frohman et al., 1993; McKay et al., 1994; Cordes and Barsh, 1994). In no case, however, have the genes been identified that directly respond to the inductive hindbrain signals and may therefore be involved in interpreting these signals. Recently, a battery of genetic loci affecting inner ear development has also been identified in the zebrafish by extensive genetic screens (Malicki et al., 1996; Whitfield et al., 1996). Further analysis and cloning of the identified mutated genes should greatly support and extend our understanding of the molecular processes governing the inner ear development in vertebrates.

We have previously isolated mouse homologs of *Drosophila* NK genes (Bober et al., 1994a). One of them, *Nkx5-1*, is specifically expressed in the otic placode and vesicle. At E8.5 to E10.5 the only additional expression domain is in the first branchial cleft (Rinkwitz-Brandt et al., 1995). In this study, we investigated whether *Nkx5-1* gene expression might depend on interactions with the hindbrain during otic placode and vesicle formation. Since the chick presents a suitable model for

investigation of tissue interactions, we first isolated the chick *Nkx5-1* homologue (*cNkx5-1*) and analysed its expression pattern. In contrast to the mouse *Nkx5-1* gene, which is also expressed in the branchial region, *cNkx5-1* transcripts were found exclusively in the otic placode and vesicle. We monitored *cNkx5-1* expression in a series of transplantation experiments to establish the spatial and temporal requirements for the *cNkx5-1* gene activation in correlation with early inner ear development. Our experiments demonstrate that the normal, regionalized *cNkx5-1* expression pattern can be established in otic vesicles after transplantation of early placodes to ectopic positions. However, as has also been documented previously, inner ear morphogenesis cannot be faithfully restored in the absence of the normal local environment (Van de Water and Represa, 1991; Swanson et al., 1990). In line with this fact, we found that the expression pattern of the paired box gene *Pax2* which is involved in the development of the cochlea (Torres et al., 1996) was not maintained in transplanted otocysts. Thus *Nkx5-1* and *Pax2*, associated with vestibular and cochlear development, respectively (Rinkwitz-Brandt et al., 1996; Hadrys et al., 1998; Torres et al., 1996), seem to differ in their susceptibility to local environmental cues.

## MATERIALS AND METHODS

### Library screening, cloning and sequencing

Chick *Nkx5-1* and *Pax2* clones were isolated by screening a genomic DNA library prepared in lambda Fix II vector (Stratagene, Heidelberg) with a hybridisation probe consisting of a 1.1 kb *StuI-EcoRI* fragment from the 3' region of the mouse *Nkx5-1* cDNA (Bober et al., 1994a) and a 1.4 kb *EcoRI* fragment of the mouse *Pax2* cDNA (Dressler et al., 1990; kindly provided by P. Gruss). Four independent overlapping lambda phages were isolated using the mouse *Nkx5-1* cDNA probe. *cNkx5-1* coding sequences were identified by Southern blot hybridisation. Following subcloning by standard procedures (Maniatis et al., 1982), sequences were determined by the dideoxy-procedure on both strands of appropriate DNA subfragments using the DNA sequencing kit (USB, Cleveland, OH). A 1.3 kb *SacI-XbaI Pax2* genomic fragment containing exon sequences was identified by sequencing, subcloned and used for generation of antisense RNA transcripts for whole-mount in situ hybridisation (see below).

### Cloning of *cNkx5-1* cDNA fragments by RT-PCR and the 5' cDNA sequences by RACE

mRNA was isolated from otic vesicles of E3 chicken embryos using the Micro-Fast Track mRNA kit (Invitrogen, Leek). 100 ng mRNA were treated with RNase-free DNaseI, phenol purified and used for the first cDNA strand synthesis with an oligo (dT) primer and AMV Reverse Transcriptase (Boehringer Ingelheim Bioproducts, Heidelberg) in a total volume of 25 µl. 1 µl of this reaction was amplified using 1 unit Taq-Polymerase (Boehringer Ingelheim Bioproducts, Heidelberg) and 25 pmol of oligonucleotide primers, which are indicated in the *cNkx5-1* sequence (Fig. 1) and correspond to two different exons of the mouse *Nkx5-1* gene. The 25 µl reaction mixture contained 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM TrisHCl pH 9.0, 0.1% Triton X-100 und 1 mM dNTP. Usually 35 cycles of amplification (denaturation for 30 seconds at 94°C, annealing 30 seconds at 55°C, elongation 60 seconds at 72°C) were performed. The amplification products were isolated from agarose gels, subcloned and sequenced.

The 5' cDNA sequences were cloned by a RACE procedure using a 5' RACE kit (GIBCO-BRL, Eggenstein). Briefly 50 ng mRNA and

2.5 pmol of the *cNkx5-1*-specific oligonucleotide (653-675): 5'-GGACTCGAGCTGGAAGACCTGG-3' were used for the first strand synthesis and tailed with an oligo d(C). Using three nested gene-specific primers: (308-327) 5'-CAGGCAGTGC GCGCTCAGAG-3', (302-322) 5'-AGTGCGCGCTCAGAGCGAACG-3', (282-302) 5'-CGCGCGCCGGGATCTCGAAG-3' cDNA fragments were synthesized in three subsequent amplification steps and identified by hybridisation with an internal *cNkx5-1*-specific oligonucleotide (239-268): 5'-GATCTCCACCTGCGAGAGG-3'. The reaction products were subcloned into the pGem-T vector (Boehringer Ingelheim Bioproducts, Heidelberg). We were not able to clone the entire 5' *Nkx5-1* cDNA probably due to the high content of GC sequences, which hindered the elongation reaction.

### Embryo preparation

Fertilized chick (*Gallus gallus*) eggs were incubated at 37.8°C and 50% humidity. Embryos were staged according to Hamburger and Hamilton (1951). Since there is a three-somite difference between each consecutive early stage, i. e. a stage 10 embryo possesses 10 somites, stage 11 embryo 13 somites etc., we subdivided the stages in + (stage 10+ means 11-somite embryo) and - (stage 10- means 9-somite embryo).

### Transplantation of otic vesicles to ectopic locations

Embryos were washed thoroughly in Hanks' Balanced Salt Solution (HBSS, Gibco) and then transverse body sections containing the region of rhombomere 5/6 and the outlying tissues were cut using tungsten needles. Otic placodes were removed using needles during brief immersion of the body sections in a solution of 1 mg/ml Dispase (Grade II, Boehringer Mannheim Biochemicals). Placodes were then washed several times in HBSS before transplantation into host embryos.

Eggs containing host embryos were windowed, several drops of Ringer solution added and embryos visualized by sub-blastodermal injection of India Ink (Pelican Fount, 1 : 15 in Ringer). The vitelline membrane was dissected away from the desired region of the embryo and a region of tissue including the ectoderm and underlying mesenchyme was removed using tungsten needles. An isolated placode was then added to the host embryo using a pipette and the tissue manoeuvred into place using needles. No care was undertaken to preserve the orientation of the transplant. Windows in operated eggs were sealed using tape (Beiersdorf) and returned to the incubator for 2 additional days in most cases. In one series of experiments, the incubation time was extended to 4 days. After incubation embryos were fixed in 4% Paraformaldehyde/PBS overnight and submitted to whole-mount in situ hybridisation. The transplantation procedures are outlined in Fig. 4.

Nearly 50% of the operated embryos were viable and showed no obvious developmental deviations. Ectopic otic vesicles were seen on the surface of about 20% of those embryos by morphological scrutiny. Another 15% developed vesicles in deeper tissue layers which were revealed on vibratome sections after in situ hybridisation. Embryos with no recognizable transplanted tissue were not included in further analysis. To ensure that otic development was not affected by the operation procedure itself sham transplantations were performed. In stage 10-12 chick embryos otic placodes were removed using tungsten needles and replaced by placodes isolated from isochronic donor quail embryos. Operated embryos were allowed to develop as usual and processed for antibody staining using a quail-specific antibody QCPN obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biology at the University of Iowa, Iowa City, IA. In successfully transplanted, normally developed embryos, the otic tissue on the operated side was entirely of quail origin and showed no abnormalities as compared to the control unoperated side. In parallel controls using chick hosts and donors, no changes in expression of *cNkx5-1* and *cPax2* genes were observed due to the operation (results not shown).

### Whole-mount in situ hybridisation

Whole-mount in situ hybridisation experiments were performed as previously described (Bober et al., 1994b). An antisense RNA probe corresponding to the 850 bp fragment from the carboxyterminal end of the *cNkx5-1* coding region (nucleotides 415-1265) was used for hybridisation. An antisense RNA *cPax2* probe was generated from the 1.3 kb *SacI-XbaI* fragment cloned into the Bluescript KS vector (Stratagene). This probe included exon sequences outside the conserved paired box and corresponded to amino acids 228-287 encoded by the mouse *Pax2* cDNA (Dressler et al., 1990).

Embryos were embedded in a mixture of 0.5% gelatine, 30% albumin, 20% sucrose and 2% glutaraldehyde and sectioned at 30 µm using the Leica Jung Autocut vibratome. Sections were photographed under a Zeiss Axiovert microscope with Nomarski optics. Whole-mount embryos were photographed under a Wild M10 stereomicroscope.

## RESULTS

### The coding sequence of the chick *Nkx5-1* gene

Fig. 1 shows the coding region of the chick *cNkx5-1* gene in comparison with the corresponding mouse cDNA. The longest product obtained by RACE procedure ended 33 bp downstream of the putative ATG initiation codon (arrow in Fig. 1). This methionine is the first ATG codon in frame with the homeodomain but the surrounding nucleotides conform poorly with the Kozak consensus sequence for translation initiation and no in frame stop codon was found in the near upstream region. Thus, the definitive NH<sub>2</sub>-terminus of the 5' *cNkx5-1* protein needs further evaluation. The derived *cNkx5-1* protein sequence revealed a homeodomain that was identical to that encoded by the mouse *Nkx5-1* gene. Sequences downstream and upstream of the homeodomain are also strongly conserved in both species with more than 80% identity. The boundary between the first and second exon is also perfectly conserved in mouse and chick (arrowhead in Fig. 1). This sequence conservation and the conserved gene structure strongly suggest that both genes represent true orthologs.

### The chick *Nkx5-1* gene is an early marker for inner ear development

Whole-mount chick embryos at stages 8 to 17 have been analysed for *cNkx5-1* expression by in situ hybridisation using digoxigenin-labeled antisense RNA probes. By this technique, *cNkx5-1* transcripts were first detectable at stage 10, at the anterior aspect of the otic placode (Fig. 2A); the rostral part of the developing inner ear continues to express the highest level of the *cNkx5-1* transcripts in the forming otic vesicle (Fig. 2B,C) until about stage 15 in the closing vesicle. The posterior part of the otic vesicle is almost devoid of *cNkx5-1* hybridisation signal as shown on vibratome sections at stage 10 (Fig. 3A-C), 12 (Fig. 3D-F), and 14 (Fig. 3G-I). At stage 17, no posterior restriction of the *cNkx5-1* expression exists (Figs 2D-F, 3J-L).

During the early morphogenetic movements of the otic placode, which result in the formation of the otic pit and later the otic vesicle, *cNkx5-1* expression displays a changing pattern. As mentioned above, *cNkx5-1* signals form a gradient along the anteroposterior axis, which originates at the most anterior part of the otic anlagen. In addition, there is a changing distribution within the mediolateral plane. At first, the *cNkx5-1* expression domain lies medially, adjacent to the neural tube (Fig. 3B,E). At stage 14, when the walls of the vesicle are

almost closed, the *cNkx5-1* signal extends to the lateral wall (Fig. 3G,H). Later, *cNkx5-1* expression is confined exclusively to the lateral wall of the otic vesicle, which does not contact the neural tissue (Figs 2E, 3J-L).

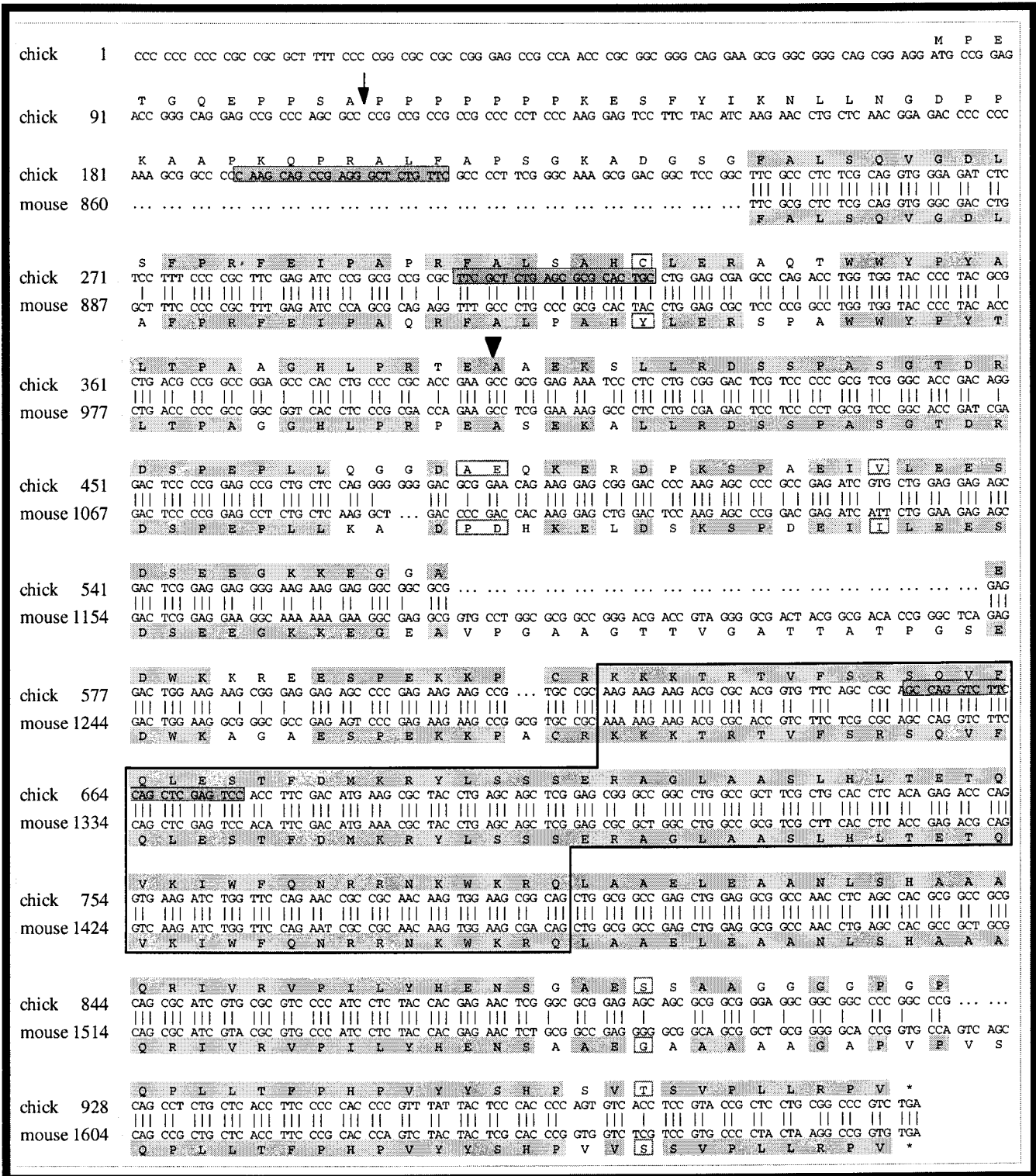
Expression of *cNkx5-1* in the inner ear anlagen was visible in the chick embryo usually after 2-6 hours of staining reaction. The only additional signal that could be observed after prolonged dye reaction (8-12 hours) was a very faint staining in the lens and in the branchial region (not shown). In contrast, in the mouse, the first branchial cleft presents a dominant, albeit transient expression domain (Rinkwitz-Brandt et al., 1995). Whether the lack of the strong *cNkx5-1* expression in the first branchial cleft in the chick is the only significant difference of *Nkx5-1* expression in these two species needs further analysis at later stages.

### *cNkx5-1* gene can be activated in otic placode transplants to anterior positions

The early *cNkx5-1* expression domain is adjacent to the neighbouring neural epithelium of the hindbrain. This prompted us to investigate whether *cNkx5-1* transcriptional activation and/or its maintenance depends on signals from the hindbrain. Placodes of various stages were transplanted to rhombomere 1/2 or midbrain levels of isochronic hosts or to the wing buds of heterochronic older hosts and *cNkx5-1* expression was analyzed in these ectopic locations (Fig. 4). Embryos, including those without macroscopically recognizable otic vesicles, were submitted to in situ hybridisation and subsequently sectioned on the vibratome to ensure that the transplants were not lost, as sometimes ectopic vesicles could only be identified after sectioning of the specimen.

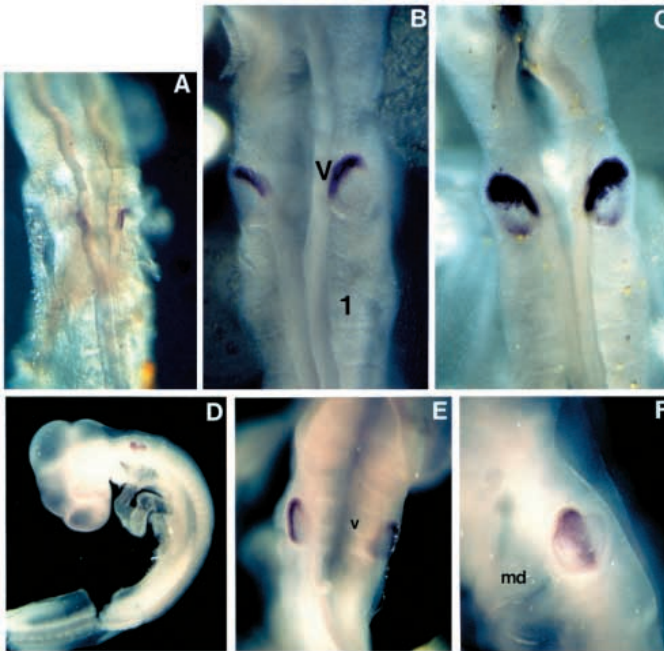
Transplantation of otic placodes to rostral locations yielded two different results depending on the age of the donor embryos. Placodes younger than stage 10 gave rise to ectopic vesicles in the majority of cases (8 out of 11 successful transplantations; see Table 1). However, these vesicles were always smaller than those of the endogenous controls and displayed no signs of continuing morphogenesis as judged by lack of endolymphatic duct formation (Fig. 5 and not shown). Half of these transplants (4) switched on the *cNkx5-1* gene, whereas the other half of ectopic vesicles (4) were negative for *cNkx5-1* gene activity. Fig. 5 presents examples of ectopic vesicles positive (Fig. 5C) and negative (Fig. 5F) for *cNkx5-1* transcripts. No clear correlation was apparent between the presence of *cNkx5-1* expression and either the location of the transplant or its age at the time of transplantation. Interestingly, one of the three transplants that did not form vesicles was slightly positive for *cNkx5-1* (not shown) indicating that *cNkx5-1* could be activated and maintained independently of vesicle formation at least for some time period. It cannot be completely ruled out that variations in ectopic *cNkx5-1* expression that were observed in transplants younger than stage 10 were caused by the operation procedure. However, in the light of results with placodes at stages 10-12 that maintained *cNkx5-1* expression very faithfully (see below), such influence is highly unlikely. Nevertheless, better orthotopic controls, which include simultaneous detection of quail marker and specific gene expression, should be established.

Transplantation of placodes at stage 10 and older to



**Fig. 1.** Sequence comparison of mouse and chick *Nkx5-1* cDNAs. The chick sequence includes only the coding regions estimated by comparison of the *cNkx5-1* genomic sequence with sequences of the RT PCR and RACE products. The 3' untranslated region of the gene is not included in the figure. The mouse cDNA sequence (Bober et al., 1994a) constitutes the region overlapping with the corresponding chick sequence. The homeodomains are boxed, the identical amino acid residues shaded grey, and the conservative exchanges are in white boxes. The asterisk marks the stop codon, the arrowhead marks the intron-exon boundary, which is conserved in both species, and the arrow indicates the end of the longest RACE product for the chick *Nkx5-1* cDNA. box. The primers used for the amplification of the RT-PCR products (192-213, 304-324 and 653-675) are shown in grey outlined boxes. Accession number for chick *Nkx5-1* is Y15989, EMBL Nucleotide sequence Database.





**Fig. 2.** *cNkx5-1* expression in E2 and E3 chick embryos. (A) Stage 10 embryo; (B,C) stage 12+ embryos; the embryo in C is stained more intensely than the one in B. (D-F) Stage 17 embryo shown from lateral (D,F) and dorsal (E) side. (E,F) Higher magnifications of D. Abbreviations: V, the fifth rhombomere; 1, the first somite; md, mandibular arch.

rhombomeres 1/2 or midbrain position resulted in the formation of ectopic vesicles in every successful operation (Table 1). In contrast to the results obtained with younger transplants in which nearly 30% (3 out of 11) of successfully transplanted placodes did not form ectopic vesicles, we never found such cases in the older group of transplants. This can be interpreted that beginning at stage 10 otic placodes are already committed to form vesicles even in anterior ectopic positions, although a permissive environment seems to be required (see below). All ectopic vesicles formed in this age category expressed *cNkx5-1* (Fig. 6; Table 1). Although significantly higher numbers of experiments were performed with placodes at stage 10 and older (21 individual successful transplantations versus 11 transplantations of placodes younger than stage 10), we never observed an ectopic vesicle lacking the *cNkx5-1* signal in this group (Table 1). This was in a sharp contrast to the results obtained with placodes younger than stage 10 in which 50% of ectopic vesicles were *cNkx5-1* negative (Fig. 5; Table 1).

The characteristic pattern of *cNkx5-1* distribution was retained in all ectopic otic vesicles which formed in the anterior locations and were *cNkx5-1* positive. As in endogenous vesicles, the *cNkx5-1* signal was always confined to the lateral wall of the ectopic otocysts (Figs 5C, 6C,F). In some cases, the ectopic expression domain appeared even broader than in the endogenous control (compare Fig. 6E to F) but in no case was *cNkx5-1* signal observed in the medial vesicle wall. This correct pattern formation was apparently not influenced by the age, shape or location of the ectopic vesicles. It was also independent of the original orientation of the placodes, since the transplanted placodes were positioned at random in the host embryo.

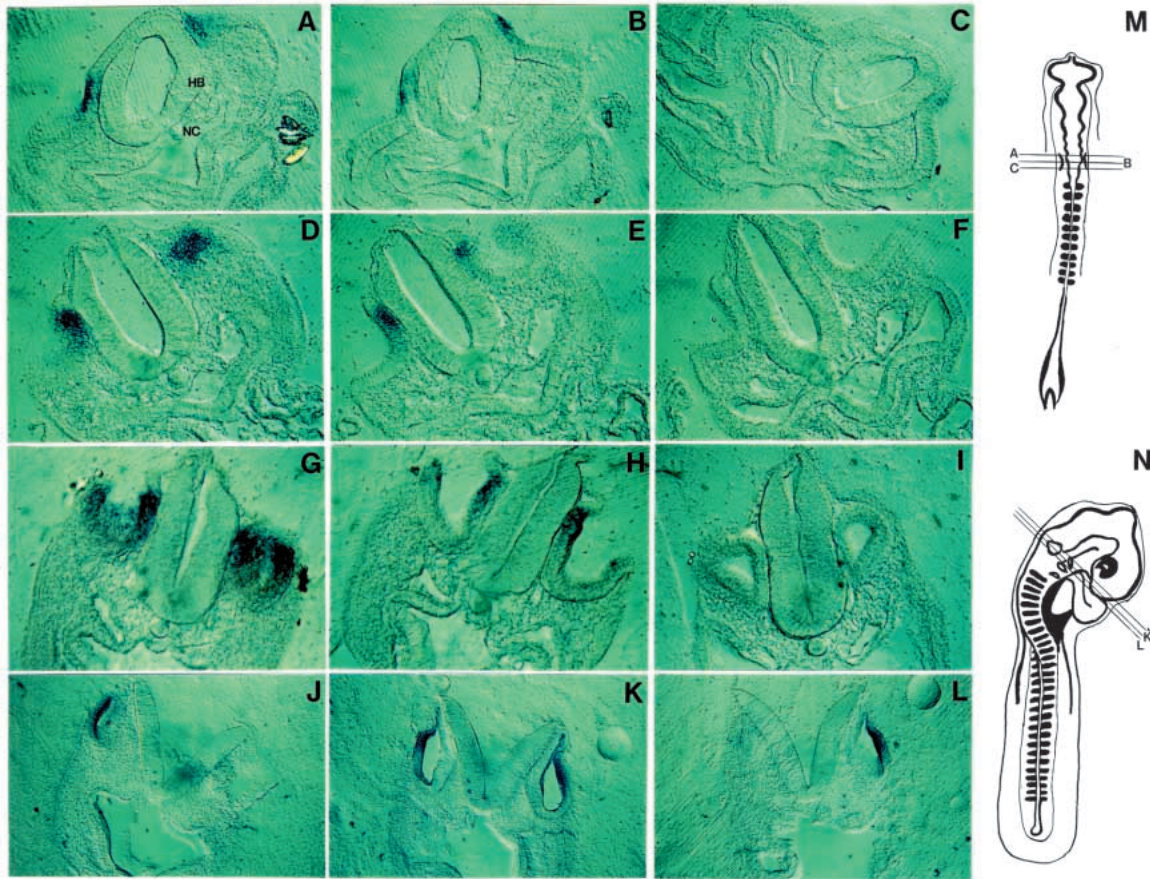
Despite the correct spatial distribution of *cNkx5-1* transcripts, morphogenesis of the inner ear did not proceed normally. Although a small percentage of the ectopic vesicles underwent initial morphogenesis as judged by the evagination of the endolymphatic duct (Fig. 6C), incubation for longer time periods revealed no subsequent morphogenetic events. Instead, the transplants remained as undifferentiated vesicles, eventually lost *cNkx5-1* expression, and later regressed (Fig. 6G,H and not shown). The endogenous control (Fig. 6G) illustrated that anlagen of individual organs as basilar papilla or semicircular canals developed normally as described for E6 chick embryos (Bissonnette and Fekete, 1996), whereas the ectopic vesicles remained arrested and lost the *cNkx5-1* signal (Fig. 6H).

#### ***cNkx5-1* expression and otic morphogenesis are not maintained in the wing buds**

The otic placode transplants into the wing buds of older hosts behaved differently from those transplanted within the head mesenchyme. In contrast to transplantations to rostral locations, we did not observe formation of ectopic vesicles from placodes younger than stage 10 (Table 1). Transplantation experiments were controlled by parallel operations using quail donors and QCPN antibody staining, which indicated the presence of the transplanted tissue (not shown). Only a small fraction (3 out of 9, Table 1) of stage 10 to 12 placodes formed vesicles in wing buds, whereas all the placodes of stage 10-12 formed otic vesicles when transplanted to the head mesenchyme (Table 1). Transplants from this age group never showed *cNkx5-1* expression in the wing buds (Table 1). Only transplanted vesicles of stage 18-19 embryos maintained their morphology and the expression of the *cNkx5-1* gene. However, the intensity of the *cNkx5-1* hybridisation signals in vesicles ectopically placed in wing buds was always weaker than that of the endogenous vesicles (data not shown). In contrast, the

**Table 1. Summary of otic placode transplantations hybridized with *Nkx5-1***

H.H. stage of the donor embryo for the otic placode isolation	H.H. stage of the host embryo	Location of the transplanted otic placode	Ectopic otic vesicle formation	<i>cNkx5-1</i> hybridisation signal	Number of experiments
8+ to 10-	9 to 10	head mesenchyme at the level of rhombomeres 1 and 2 or the midbrain	8	4	11
10 to 12	10 to 12	head mesenchyme at the level of rhombomeres 1 and 2 or the midbrain	21	21	21
8 to 9+	18 to 21	wing buds	0	0	5
10 to 12	18 to 21	wing buds	3	0	9
18 to 19	18 to 21	wing buds	5	4	5



**Fig. 3.** Vibratome sections of *cNkx5-1* hybridised embryos. (A-C) Anterior-to-posterior 30 µm sections at the otic placode region of the stage 10 embryo showed as a whole mount in Fig. 2A. (D-F) Anterior-to-posterior 30 µm sections from the otic pit region of the stage 12+ embryo showed as a whole mount in Fig. 2B. (G-I) Anterior-to-posterior sections (30 µm) at the level of the open otic vesicle of a stage 14 embryo. (J-L) Anterior-to-posterior sections (30 µm) from the closed otic vesicle region of the stage 17 embryo showed as a whole mount in Fig. 2D-F. (M,N) The planes of sections shown in A-C and J-L, respectively. Abbreviations: hb, hindbrain; nc, notochord.

signals of ectopic otic vesicles in the head mesenchyme always matched the intensity of the corresponding endogenous controls (Fig. 6A-F).

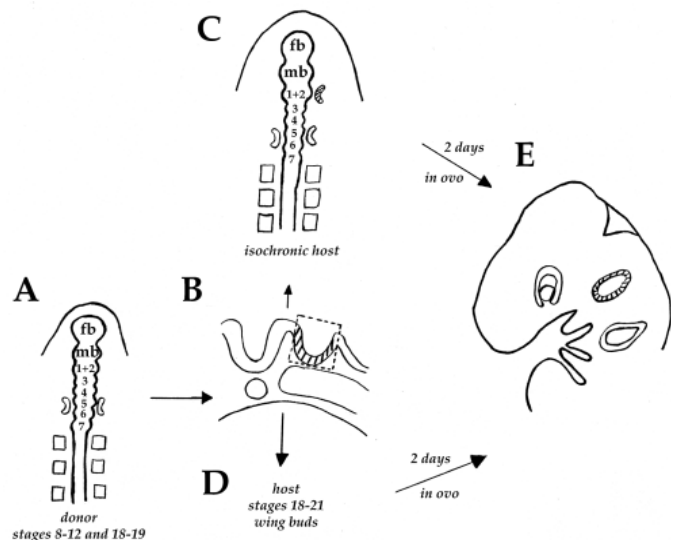
**Early expression of *Pax2* gene in the chick inner ear**

The reconstitution of endogenous *cNkx5-1* expression pattern in ectopically formed otocysts suggested that at least some aspects of molecular patterning can be preserved independently of the original environment. However, as pointed out above, placodes or vesicles never gave rise to normal inner ear morphogenesis after transplantation. We were interested to investigate whether other genes expressed in defined regions of the otocyst behaved similarly to *cNkx5-1* in an ectopic location. We chose *Pax2* as a gene that shows an

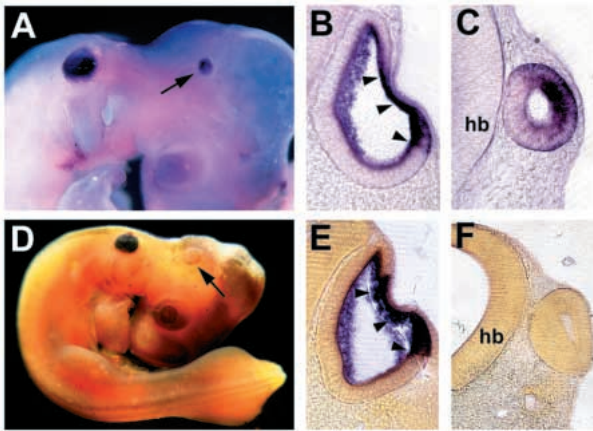
almost complementary transcript distribution to *Nkx5-1* in the mouse otocyst and inner ear with a clear restriction to the prospective cochlear region (Rinkwitz-Brandt et al., 1995, 1996).

**Fig. 4.** A scheme illustrating the transplantation experiments.

(A) Transverse body sections were made of donor embryos of stages 8-12 and 18-19 at the level of the 5th and 6th rhombomeres. (B) Placodes were released from neighbouring tissues in dispase solution and (C) inserted either into the more anterior locations of the age matched hosts or (D) into the wing buds of hosts at stage 18-21. (E) The host embryos were screened for ectopic otic vesicles and *cNkx5-1* expression after 2 days of further development. Abbreviations: fb, forebrain; mb, midbrain; 1-7, rhombomeres 1-7.





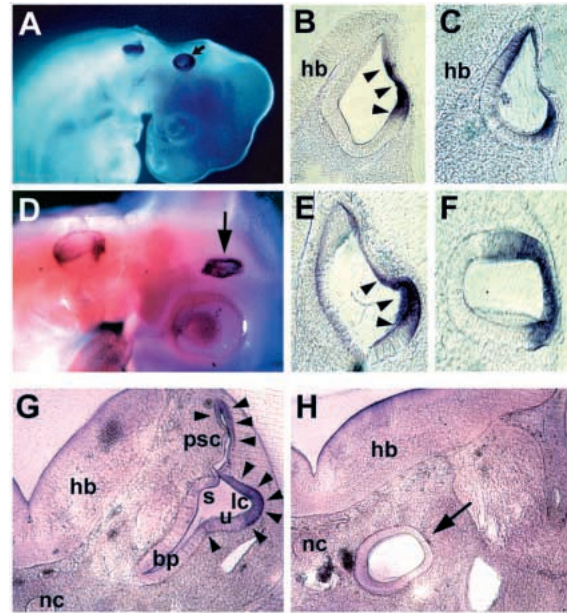


**Fig. 5.** *cNkx5-1* expression in ectopic and endogenous otic vesicles transplanted before stage 10. Embryos hybridised with a *cNkx5-1* digoxigenin antisense RNA probe and representative for two groups of otic placode transplants younger than stage 10 are shown. For each group, one whole-mount embryo with an ectopic otocyst positive (A) and negative for *cNkx5-1* (D) is presented. Transverse sections through endogenous (B,E) and ectopic (C,F) vesicles document positive *cNkx5-1* signal in the controls (B,E) and in one group of transplants (C) whereas the second group lacks such signal (F). *cNkx5-1* signal was confined to the lateral wall of the ectopic otocyst (C), similarly to the endogenous situation (B,E). Note that dorsal evagination indicating formation of the endolymphatic duct is clearly visible in control (B,E) but not in ectopic vesicles (C,F). Arrows point to the ectopic otic vesicles, arrowheads indicate the size of the endogenous *cNkx5-1* expression domain. Abbreviation: hb, hindbrain.

*cPax2* is activated early during otic development and its transcripts can be detected by in situ hybridisation already during placodal stages (Fig. 7A). In contrast to *cNkx5-1*, *cPax2* expression in the otic placode does not show any rostrocaudal restriction but is present along the entire length of the placode (not shown). Laterally the *cPax2* expression domain appears wider than that of *cNkx5-1* and extends into the surface ectoderm beyond the placode itself (compare *cPax2* expression in the 12-somite-stage placode shown in Fig. 7A to *cNkx5-1* expression at 10-somite-stage placode in Fig. 3A). During vesicle formation, *cPax2* expression undergoes regionalization and concentrates to the dorsomedial wall (Fig. 7C). This is different from the *Pax2* expression pattern described in the mouse where the *Pax2* domain is placed in the ventromedial wall of the otocyst (Nornes et al., 1990; Rinkwitz-Brandt et al., 1995, 1996). Beside this slight spacial difference *Pax2* expression domains are generally conserved between mouse and chick (Fig. 7D and not shown).

#### Ectopic otocysts do not restore normal regionalization of *cPax2* transcripts

In order to analyze the dependence of *cPax2* transcript distribution on local cues, a total of 20 transplantations of otic placodes at stage 10-12 to the rostral locations were analyzed for *cPax2* expression (Table 2). As already described, such placodes always gave rise to ectopic vesicle formation after healing of the transplanted tissue. However, *cPax2* expression in those vesicles differed significantly in its response to transplantation from that observed for *cNkx5-1* transcripts.

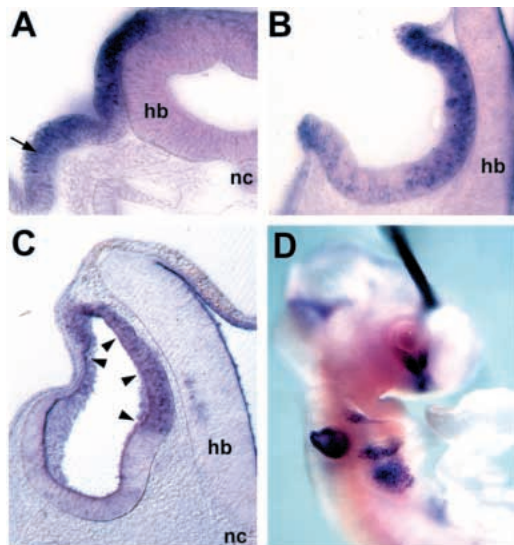


**Fig. 6.** *cNkx5-1* expression in ectopic and endogenous otic vesicles transplanted at stage 10-12. Examples of embryos after transplantation of otic placodes at stage 10-12 to (A-C) the level of rhombomere 1/2 or (D-F) the midbrain and incubated for 2 days are shown. (G,H) Transverse sections of the endogenous (G) and ectopic (H) otocyst obtained in a similar transplantation experiment but incubated for 4 days. All specimens were hybridised with a *cNkx5-1* digoxigenin antisense RNA probe. Note the correct spatial *cNkx5-1* expression pattern restored in ectopic vesicles in C and F and a lack of such pattern as well as the most of the signal in a longer incubated otic vesicle (H). Arrows point to the ectopic otic vesicles, arrowheads indicate the size of the endogenous *cNkx5-1* expression domain. Abbreviations: bp, basilar papilla; hb, hindbrain, lc, evaginating lateral semicircular canal, nc, notochord, psc, primordium of the anterior and posterior semicircular canals, s, sacculus, u, utriculus.

According to the intensity of *cPax2* expression in ectopic vesicles, two groups of results were obtained. In slightly more than one half of vesicles (11 out of 20, Table 2), the level of *cPax2* expression was comparable to or only slightly lower than that observed in the endogenous otocysts of the same embryo. Examples of such ectopic vesicles are shown in Fig. 8C and F-H. The other half (9 out of 20, Table 2) showed significantly weaker or no *cPax2* expression, whereas expression level in the endogenous control remained normal (Fig. 8E,I). These results indicate that the regulation of *cPax2* mRNA levels is much more sensitive to signals from the local environment than that of *cNkx5-1*.

Another striking difference between *cNkx5-1* and *cPax2* transplants concerned the specific regionalized distribution of transcripts. Whereas ectopic otic vesicles reconstituted quite faithfully the endogenous *cNkx5-1* expression pattern, this was not the case for *cPax2* transcripts. Ectopic otocysts exhibited a very variable regional *cPax2* expression and often switched on *cPax2* in the lateral wall instead of the medial wall which is the normal region of *cPax2* transcripts (compare Fig. 7C and 8B,D for endogenous and Fig. 8C and E-I for ectopic *cPax2* expression). As already mentioned above, the orientation of transplanted placodes was random. Therefore, changes



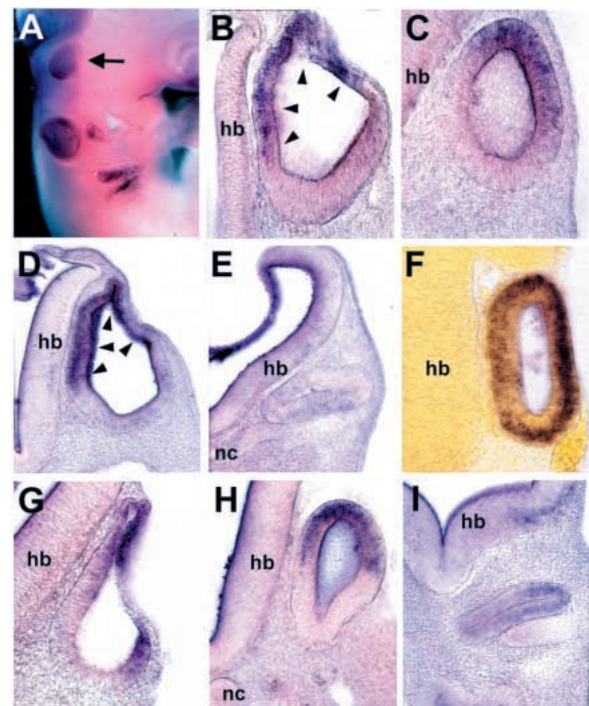


**Fig. 7.** *cPax2* expression in E2 and E3 chick embryos. (A-C) Transverse sections through the otocysts of stage 11– (12 somites; A), stage 15 (25 somites; B) and stage 21 (E3.5; C) embryos. (D) A whole-mount embryo of stage 21 (E3.5). Embryos were hybridized with a *cPax2*-specific antisense RNA probe labeled with digoxigenin. Arrow in A points to *cPax2* expression which extends laterally into the surface ectoderm. Arrowheads indicate the size of the *cPax2* domain within the closed otocyst (C). Abbreviations: hb, hindbrain; nc, notochord,

observed in *cPax2* expression cannot be explained by preservation or rotation of the original orientation. Moreover, several vesicles were found that showed *cPax2* expression throughout the otic vesicle and not confined to only one side (see Fig. 8F). To summarize, our results demonstrate that two regulatory genes behave differently after transplantation of otic placodes to more rostral locations. While the distribution and accumulation of *cNkx5-1* transcripts was readily maintained in ectopic otocysts for extended periods of time (up to two days), the correct spatial distribution as well as the level of *cPax2* transcripts appeared much more susceptible to local influences.

## DISCUSSION

In this study, we have analysed the development of otic placodes in ectopic locations in the chick and correlated it with expression of two otic-specific regulatory genes, *cNkx5-1* and *cPax2*. Our experiments confirm some of the previously published data on amphibia that ear placodes become determined to some extent already by the early neurula stage (Jacobsen, 1963). In addition, we demonstrate that such



**Fig. 8.** *cPax2* expression in ectopic and endogenous otic vesicles transplanted at stage 10–12. Two different kinds of results concerning the *cPax2* signal intensity are exemplified in B–C and D–E. (A) The operated side of an embryo, which showed levels of ectopic *cPax2* expression comparable to endogenous control is shown. (B,C) Transverse sections through the endogenous (B) and ectopic (C) otocysts. Transverse sections through endogenous (D) and ectopic (E) vesicles of another embryo demonstrate only little ectopic *cPax2* expression (E) as compared to the endogenous control in the same embryo (D). (F–I) Transverse sections through different ectopic vesicles hybridised with the *cPax2* probe and showing relatively strong (F–H) or weaker (I) *cPax2* expression. Note that no restoration of the regionalized *cPax2* expression can be observed in any of the ectopic vesicles. Arrow points to the ectopic vesicle in A. Arrowheads indicate the size of the *cPax2* domain within the endogenous otocysts (B,D). Abbreviations: hb, hindbrain; nc, notochord.

determination may include the vestibular but not the cochlear pattern as exemplified by *cNkx5-1* and *cPax2* expression, respectively.

### Otic placodes younger than stage 10 (5–9 somites) can undergo the initial steps of morphogenesis in permissive environment

In contrast to previous observations in the chick (Waddington,

**Table 2. Summary of otic placode transplantations hybridized with *Pax2***

H.H. stage of the donor embryo for the otic placode isolation	H.H. stage of the host embryo	Location of the transplanted otic placode	Ectopic otic vesicle formation	<i>Pax2</i> hybridisation signal strong/weak*	Number of experiments
10 to 12	10 to 12	head mesenchyme at the level of rhombomeres 1 and 2 or the midbrain	20	11/9	20

\*Strong signal was counted when expression intensity in ectopic vesicle was at the similar level as the endogenous control; weak signal was either significantly weaker than the control or not recognizable at all.

1937), we found that placodes younger than stage 10 (9 or fewer somites) were able to form vesicles in an ectopic environment. This discrepancy to results obtained by Waddington is probably due to differences in the applied techniques and different transplant locations, as Waddington transferred the placodes into the amniocardiac vesicle. Our experiments demonstrate that the location of the transplant is important for vesicle formation. Placodes younger than stage 10 never formed vesicles when transplanted to the wing buds. This observation is in line with the results of Swanson et al. (1990), who found that only placodes older than stage 13 could develop and even differentiate to various inner-ear-specific cell types within the wing buds. However, experiments on salamanders suggested that ear placodes may be determined to some extent already at early neurula stages, as reversing the anteroposterior axis of the brain plate did not affect the site of ear formation (Jacobson, 1963). Similarly, a significant fraction of *Xenopus* ear placodes at the neural plate stage were able to form otocysts under neutral explant conditions (Gallagher et al., 1996). Furthermore, the observation that young otic placodes may undergo initial morphogenesis, i. e. form otic vesicles without the influence from the neighbouring rhombomeres 5/6 is strengthened by the fact that, in some mouse hindbrain mutants such as Kreisler or *Hoxa-1*<sup>-/-</sup> mice, the original placode and otic vesicle formation is not affected (Mark et al., 1993; Frohman et al., 1993; McKay et al., 1994; Cordes and Barsh, 1994). However, as shown in these mutants and demonstrated by our transplantations, further steps of inner ear morphogenesis critically depend on local signals.

Taken together, it appears likely that determination of ear placodes takes place very early and the subsequent vesicle formation can proceed given a permissive environment. Such environment seems to be provided by rostral tissues but not by amniocardiac vesicles (Waddington, 1937) or wing buds (Swanson et al., 1990 and this report). However, the exact source of the ear inducing and/or patterning signals remains to be defined. Gallagher et al. (1996) have shown in studies on *Xenopus* that the anterior ventral ectoderm displays a similar ability to respond to ear induction as the prospective otic ectoderm, whereas the posterior ventral ectoderm loses this ability very early in development. Since anterior and posterior ectoderm are clearly different, it cannot be excluded that the head ectoderm exerts the permissive or to some extent even instructive influences. The same might be true for the rostral hindbrain and midbrain or the underlying mesodermal tissues. In contrast, the wing bud either lacks the permissive environment or sends inhibitory signals. We are currently analysing the determination status of chick placodes in collagen gel explant cultures to estimate more exactly the timing of the otic placode specification and its correlation to specific gene activities. We also aim to define the tissue(s) that is the source of ear-forming bias imparted to the ectoderm. The bias formation is well established for the lens induction process (Grainger, 1992) and was also postulated for the ear (Gallagher et al., 1996).

#### **Otic placodes are determined for activation and regionalization of *cNkx5-1* expression around stage 10 but need permissive environment for their maintenance**

Despite the variety of experimental data on the inner ear

induction and specification, we still understand very little about it in molecular terms. Therefore, we monitored expression of *cNkx5-1*, a gene uniquely expressed in the ear placode beginning at the 10-somite stage. Our results have demonstrated that, beginning at stage 9 or even slightly earlier, chick otocysts could not only be formed ectopically but were also able to generate a specific pattern of *cNkx5-1* expression. This was an unexpected observation since in vitro experiments on explanted mouse otocysts suggested that a hindbrain influence is required for patterning and differentiation until relatively late stages (Van De Water, 1983). Together with the fact that *cNkx5-1* expression was not maintained in the wing environment, these experiments strongly suggest that specific supporting signals are present in rostral locations. It is still not clear whether the onset of *cNkx5-1* transcription requires external signals, i. e. those from the hindbrain, or whether intrinsic placode signals, which are activated once the 'placode forming program' has been initiated, are responsible for *cNkx5-1* activation. It is also possible that different signals may be required for the first gene activation and for the subsequent regionalization of *cNkx5-1* transcripts.

#### **Specific expression pattern of regulatory genes are established independently during early stages of the otic development and seem to require different signals as documented by differences obtained by *cNkx5-1* and *cPax2* pattern reconstitution in ectopic locations**

Most interestingly, another regulatory gene, *cPax2*, behaves very differently than *cNkx5-1* and appears to be more dependent on local signals. Whereas all ectopic vesicles older than stage 10 were *cNkx5-1* positive and restored their original regionalized expression pattern, only approximately a half of vesicles obtained in identical transplantation experiments retained the endogenous level of *cPax2* transcriptional activity. *cPax2* signals in the remaining vesicles were always weaker than the corresponding endogenous control and the specific, regionalized *cPax2* transcript distribution was never restored. It remains to be elucidated which mechanisms are responsible for the observed differences in the ability to reconstitute patterning in ectopic otic vesicles. It might be significant that the two genes analyzed here are involved in the control of morphogenesis of different parts of the inner ear. In the mouse, *Pax2* is responsible for the outgrowth of the cochlea (Torres et al., 1996) whereas *Nkx5-1* is required for the formation of the semicircular canals, as has been recently demonstrated by our group (Hadrys et al., 1998). It might well be that vestibular and cochlear parts of the inner ear respond to different signals and develop largely independently as already suggested by others (Fekete, 1996). Our experiments suggest either that unknown signals influencing *cNkx5-1* expression in the ectopic placodes are present in anterior locations or that vesicles of stages 10-12 are already able to maintain some of the intrinsic patterning features. However, the random orientation of the transplants in our experiments suggests that, in the rostral locations, either lateral supportive, or medial inhibitory, or both, signals exist that influence the *cNkx5-1* expression. Such signals are obviously not sufficient for the regulation of *cPax2* transcription. Interestingly, some signalling molecules such as *wnt3a* and *wnt4* (Moon, 1993), *FGF3* (McKay et al., 1996) or *BMP4*, *BMP5* and *BMP7* (Oh et al., 1996; Wu and Oh, 1996)

have been already reported to be unequally distributed in the otic vesicle. Further experiments are necessary for the identification of the functional significance of these signals. The influence from the hindbrain is a good candidate for an external regulatory signal because hindbrain signals are important not only for the primary induction but also for the further steps of the otic determination and/or differentiation (Li et al., 1978; Van de Water, 1983).

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