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PATTERNS & PHENOTYPES

Expression of Avian *prickle* Genes During Early Development and Organogenesis

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Chicken homologues of *prickle-1* (*pk-1*) and *prickle-2* (*pk-2*) were isolated to gain insight into the extent of planar cell polarity signaling during avian embryogenesis. Bioinformatics analyses demonstrated homology and showed that *pk-1* and *pk-2* exhibited conserved synteny with *ADAMTS20* and *ADAMTS9*, GON-related zinc metalloproteases. Expression of *pk-1* and *pk-2* was established during embryogenesis and early organogenesis, using in situ hybridization and sections of chicken embryos. At early stages, *pk-1* was expressed in Hensen's node, primitive streak, ventral neural tube, and foregut. In older embryos, *pk-1* transcripts were detected in dorsolateral epithelial somites, dorsomedial lip of dermomyotomes, and differentiating myotomes. Furthermore, *pk-1* expression was seen in lateral body folds, limb buds, and ventral metencephalon. *pk-2* was expressed in Hensen's node and neural ectoderm at early stages. In older embryos, *pk-2* expression was restricted to ventromedial epithelial somites, except in the most recently formed somite pair, and limb bud mesenchyme. *Developmental Dynamics 237:1442–1448, 2008.*

Key words: prickle; planar cell polarity; PCP; Primitive streak; Somite; Limb bud; ADAMTS

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INTRODUCTION

During invertebrate development, diffusible ligands are interpreted by planar cell polarity (PCP) signaling to coordinate morphogenetic events. In *Drosophila*, PCP establishes the plane of cell orientation in the developing cuticle, eye, and wing by means of the distal and proximal subcellular localization of several key proteins, including prickle (pk; Lawrence et al., 2007; Seifert and Mlodzik, 2007). In the primitive chordate, *Ciona savignyi*, *Pk* regulates cell intercalation of notochord cells (Jiang et al., 2005). Vertebrate *Pk* homologues coordinate the morphogenetic events of convergent extension movements and cell orientation in the inner ear (Carreira-Barbosa et al., 2003; Veeman et al., 2003; Deans et al., 2007). Tissue responses to pk-mediated PCP signals are caused by a combination of polarized cytoskeletal and cell adhesion rearrangements by means of the activities of Jun kinase, members of the Rho family of small GTPases, and calcium signaling (Classen et al., 2005; Gibson et al., 2006; Shimada et al., 2006; Lecuit and Lenne, 2007; Slusarski and Pelegri, 2007).

The complete extent of PCP signaling during vertebrate development is not well understood. Furthermore, while *pk* function has been well characterized in the context of PCP signals, it is becoming clear that *pk* homologues have functions beyond establishing PCP. For example, *pk-1* antagonizes Wnt/β-catenin signals in vitro by targeting Dsh for degradation (Veeman et al., 2003; Chan et al., 2006). In addition, recent in vivo studies have identified *pk-1* as a regulator of notch activity. In the *Drosophila* eye, PCP signals form a feedback loop that regulates Notch and Delta signaling (Cooper and Bray, 1999; Fanto and Mlodzik, 1999). The *pk* mutant, $pk^{pk/sple}$ ectopically activates notch activity in the *Drosophila* eye (Strutt,

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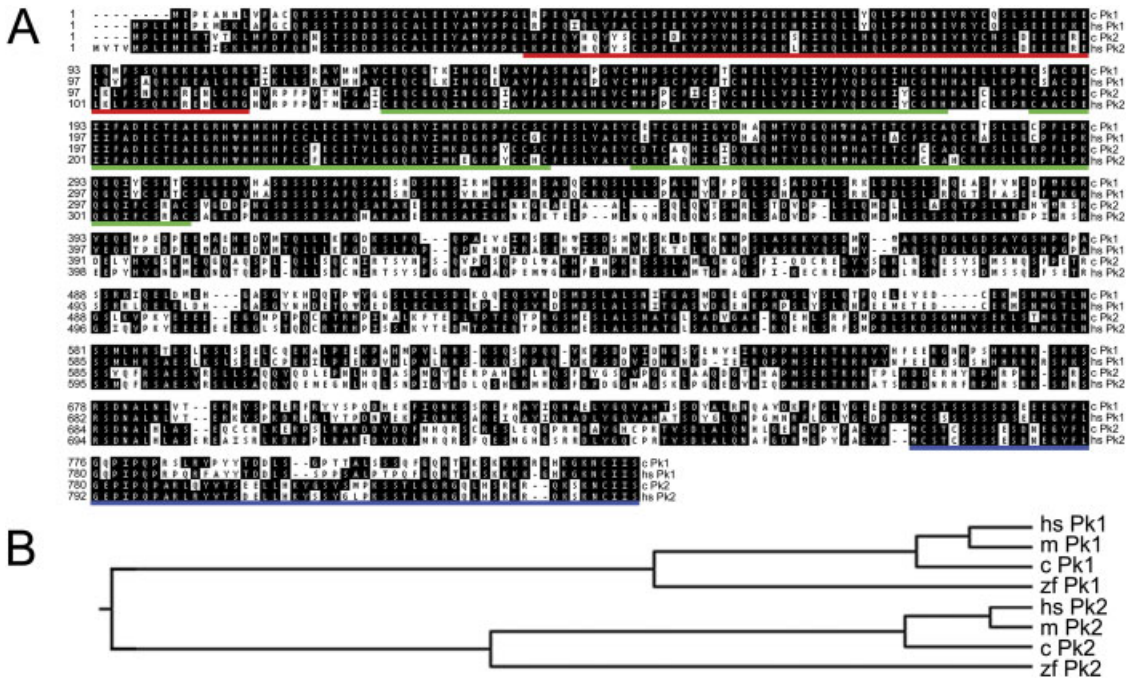

Fig. 1. Comparison of *pk-1* and *pk-2* homologues. **A:** Alignment of the predicted amino acid sequence for the chicken *pk-1* and *pk-2* genes. PET (red), LIM (green), and PKH (blue) domains are indicated with consensus matches (black shade) for chick and human homologues of *pk-1* and *pk-2*. **B:** A phylogenetic tree shows the degree of similarity of *pk-1* and *pk-2* human (hs), mouse (m), chicken (c), and zebrafish (zf) orthologues.

2002), and *pk* homologues facilitate the nuclear translocation of charlatan/NRSF/REST, a repressor of the Notch ligand, Delta (Shimojo and Hersh, 2003; Shimojo and Hersh, 2006; Tsuda et al., 2006).

During the first 4 days of chicken development, the embryo undergoes extensive remodeling, both during the establishment of the three germ layers (ectoderm, mesoderm and endoderm) and during tissue formation. To investigate the possible extent of prickle function in PCP and other signaling pathways, we examined the expression patterns of the chicken *pk* homologues, *pk-1* and *pk-2* during this period of embryogenesis.

RESULTS AND DISCUSSION

Comparison of Chicken pk-1 and pk-2 Proteins

Polymerase chain reaction (PCR) primers were designed, using the EnsemblGenomeBrowser(www.ensembl.org), to amplify the predicted coding sequences for the chicken homologues of *pk-1* and *pk-2* (see the Experimental Procedures section). While *Drosophila pk* is alternatively spliced to produce *pk*, *pkM*, and *sple* isoforms (Gubb et al., 1999), there is currently no evidence from Northern blots or expressed sequence tag databases that vertebrate *pk-1* or *pk-2* are alternatively spliced (Deans et al., 2007). Based on the predicted protein sequences, chicken *pk-1* and *pk-2* encode 828 amino acid and 832 amino acid proteins, respectively. Phylogenetic analyses revealed strong sequence similarities of chicken *pk-1* with human (88%) and zebrafish (64.3%) homologues. Similarly, chicken *pk-2* had strong sequence similarity with human (85.5%) and zebrafish (56.5%) homologues. In addition, our phylogenetic analyses indicated that the identities of mouse *pk-1* and *pk-2* were interchanged and this was adjusted (Fig. 1; Table 1; Katoh and Katoh, 2003).

Structural analyses showed that chicken pk-1 and pk-2 proteins are composed of an N-terminal PET domain, three central LIM domains, and a C-terminal PKH domain. The LIM motifs (Lin-11 Isl-1 Mec-3; InterPro accession IPR001781) are cysteine-rich, contain zinc-binding protein domains and commonly mediate protein–protein interactions (Michelsen et al., 1993; Dawid et al., 1998; Gubb et al., 1999). Emerging evidence suggests that the LIM motif is a hallmark of proteins that associate with both the actin cytoskeleton and the transcriptional machinery (Kadrmas and Beckerle, 2004). The N-terminal PET domain combines with the three LIM domains during interactions with other proteins (Gubb et al., 1999). The PKH domain contains a CaaX-motif prenylation site that determines protein–protein and protein–membrane interactions (Desnoyers and Seabra, 1998; Maurer-Stroh et al., 2003). Biochemical and mutagenesis studies of PRICKLE1 and *Drosophila* pk have demonstrated that this CaaX motif is a farnesylation that regulates nuclear/nuclear membrane localization (Shimojo and Hersh, 2003, 2006; Veeman et al., 2003). To further characterize the amino acid sequence of chicken pk-1, we used a transmembrane topology prediction method (MEMSAT3, http://bioinf.cs.ucl.ac.uk/psipred/) (Jones et al., 1994). The method predicted a transmembrane helical domain between 801 and 818 amino acids of chicken pk-1, adjacent to the CaaX motif within the PKH domain. The prediction of a transmembrane helical domain at the C-termi-

TABLE 1. Protein and cDNA Sequence Accession Numbers for prickle-1 and prickle-2

Gene	Species	Protein Accession NCBI and Ensembl	cDNA Accession NCBI and Ensembl
Prickle-1	*Gallus gallus*	ENSGALP00000015542	ENSGALG00000009556
Prickle-1	*Homo sapiens*	NP_694571.1 ENSP00000345064	NM_153026 ENSG00000139174
Prickle-1 (Predicted)	*Mus musculus*	AAI17894 ENSMUSP00000049204	BC117893 ENSMUSG00000030020
Prickle-1	*Danio rerio*	NP_899185 ENSDARP00000059513	NM_183342.2 ENSDARG00000040649
Prickle-2	*Gallus gallus*	ENSGALP00000011832	ENSGALG00000007332
Prickle-2	*Homo sapiens*	NP_942559.1 ENSP00000295902	NM_198859 ENSG00000163637
Prickle-2	*Mus musculus*	AAI45755 ENSMUSP00000032093	NM_001033217.1 ENSMUSG00000030020
Prickle-2	*Danio rerio*	NP_899186.1 ENSDARP00000054743	NM_183343 ENSDARG00000037593

nus of chicken pk-1 suggests that the protein can function as an integral protein. Four other C-terminal domains of PRICKLE1 regulate protein localization (Shimojo and Hersh, 2006). We used bioinformatics to confirm that these domains were conserved in chicken pk-1 and pk-2 (www.psort.org) (Zhou et al., 2004; Xue et al., 2005). In addition, chicken pk-1 contained three predicted nuclear localization signals (NLS) and a cyclic AMP-dependent protein kinase A (PKA) phosphorylation site, which were conserved with PRICKLE1. However, chicken pk-2 contained only one predicted NLS domain, which was shared by chicken pk-1 and PRICKLE1. Moreover, a leucine zipper pattern adjacent to the final LIM domain was predicted in chicken pk-2. When taken together, bioinformatic analyses of chicken pk-1 and pk-2 indicate that the N-termini bind to protein targets, while the C-termini regulate the localization of the putative protein complex.

Genomic Organization of Chicken pk-1 and pk-2

To characterize the chicken *pk* homologues further, the genomic organization of these two genes was examined. Chicken *pk-1* was located on contig 6.521 of chromosome 1 and was syntenic with q12 of human chromosome 12 (PRICKLE1) and D3 of mouse chromosome 6 (Prickle1). Similarly, chicken *pk-2* was located on contig 16.44 of chromosome 12 and syntenic with p14.1 of human chromosome 3 (PRICKLE2) and F1 of mouse chromosome 15 (Prickle2).

Of interest, chicken *pk-1* and *pk-2* shared similar PET/LIM domains and synteny with chicken *testin* (ENSGALG00000009398; NP_989954.1) and chicken *dyxin/LMCD1* (ENSGALG00000008349). Testin associates with the cytoskeleton, whereas dyxin acts as a transcriptional cofactor. Neither protein contains a PKH domain (Katoh and Katoh, 2003; Drusco et al., 2005; Rath et al., 2005). Immediately upstream of *pk-1* and *pk-2* in the chicken genome were *ADAMTS20* and *ADAMTS9*. ADAMTS enzymes are secreted zinc metalloproteases that digest extracellular matrix substrates, and ADAMTS20 and ADAMTS9 are highly conserved GON-related enzymes (Somerville et al., 2003;

Fig. 2. Expression of *pk-1* in stage XII and Hamburger and Hamilton stage (HH) 3–HH20 chick embryos. Stages are indicated in each panel. **A–F:** Stage XII to HH9 whole-mount embryos. **C′–E‴:** Paraffin sections through the embryos shown in C–E, the approximate levels of sections are indicated by a black, horizontal line; white arrowheads indicate Hensen's node (C′,E‴), primitive streak (C″,E″), or notochord (D′,E″); black arrowheads indicate neuroectoderm (C″,D″,E‴) and floor plate (D′,E″); asterisk indicates foregut (E′). **G–J:** Transverse sections (10 μm) through the embryo in F. Black lines in F indicate the level of the corresponding sections. **K:** Dorsal view of a HH17 embryo. **L–O:** Transverse sections (10 μm) through the embryo in K. Black lines in (K) indicate the level of the corresponding sections. M,O: Higher magnification of the regions indicated in L,N by a black box. Arrow in M indicates dorsomedial lip (dml) of the dermomyotome. Arrow in O indicates the floor plate (fp). **P:** Lateral view of a HH20 embryo. **Q:** Transverse section through the forelimb of an HH20 embryo. **R:** Frontal section through the trunk of a HH20 embryo. White lines in P indicate the level of the corresponding sections. ao, dorsal aorta; dm, dermomyotome; dml, dorsomedial lip; ds, dorsal epithelial somite; fg, foregut endoderm; flb, forelimb bud; fp, floor plate; Hn, Hensen's node; lpm, lateral plate mesoderm; lbf, lateral body fold; my, myotome; me, mesencephalon; nc, notochord; nf, neural fold; np, neural plate; nt, neural tube; sc, sclerotome; so, somite. Scale bars = 100 μm.

Fig. 3. Expression of *pk-2* in Hamburger and Hamilton stage (HH) 3–HH20 chick embryos. HH stages are indicated in each panel. **A–E:** Stage XII and HH4 to HH11 whole-mount embryos. **B′–D″:** Paraffin sections of embryos shown in B–D, the approximate levels of sections are indicated by a black, horizontal line, white arrowheads indicate Hensen's node (B′,C″,D″) or the primitive streak (B″); black arrowheads indicate neuroectoderm (C′,D′,B″,C″). **F–I:** Transverse sections (10 μm) through the embryo in E. The black lines in E indicate the level of the corresponding section. **J,K:** Lateral views of HH16 and HH20 chick embryos. **L–N:** Higher magnification views of the forelimb, tail bud, and hindlimb buds of an HH20 embryo. **O:** Transverse section through the forelimb bud of an HH20 embryo. Scale bars = 100 μm. aer, apical ectodermal ridge; fl, forelimb; hl, hindlimb; vs, ventral somite; all other abbreviations as in Figure 1.

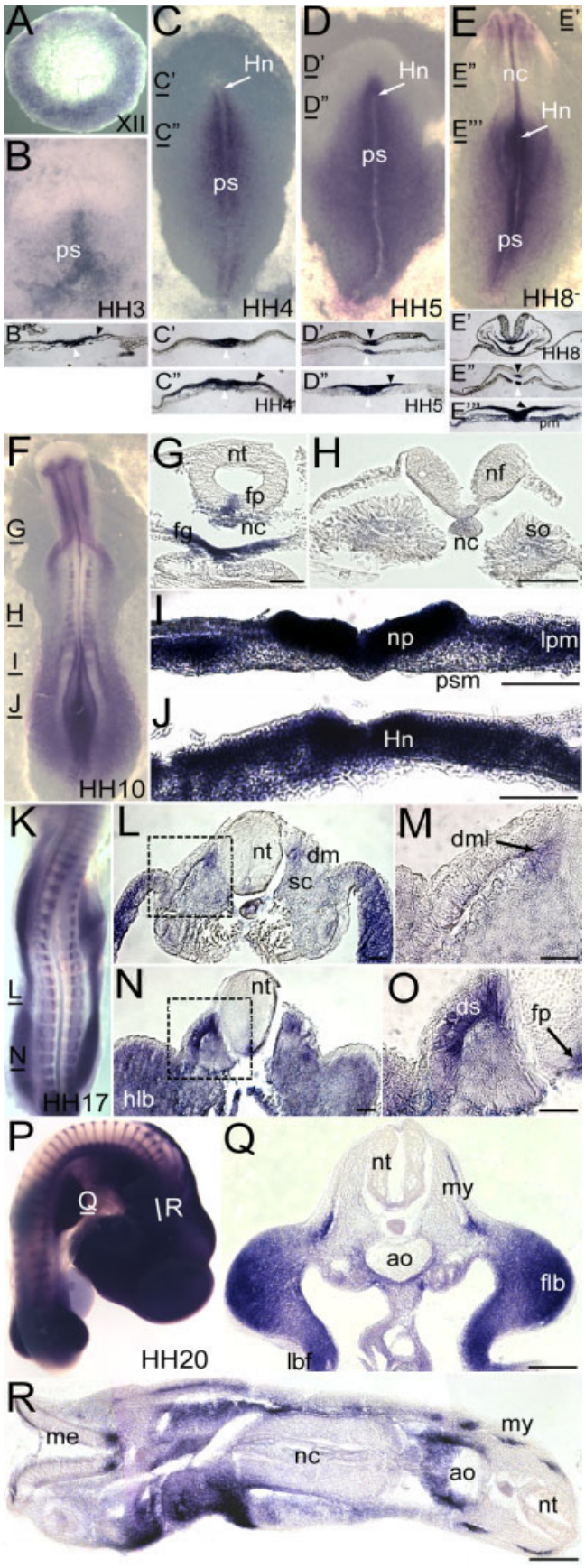
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Fig. 2.

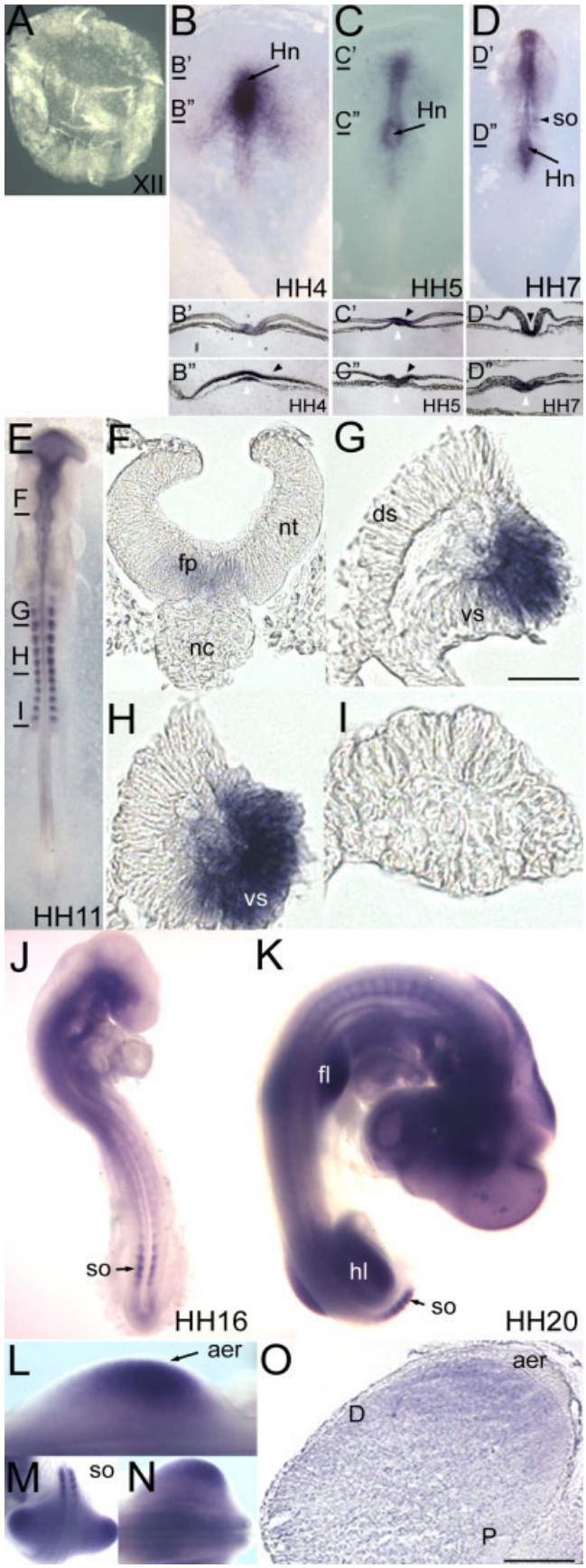
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Fig. 3.

Porter et al., 2005). The genomic organization of *pk* and *ADAMTS* homologues was conserved from *Caenorhabditis elegans* to Human. For example, *GON-1*, the *C. elegans* orthologue of *ADAMTS9* and *ADAMTS20*, was found to be syntenic with the *C. elegans pk* orthologue, *ZK381.5*. This remarkably conserved genomic environment raises the intriguing possibility that this cluster of genes is coordinately regulated during development by common regulatory elements (Sproul et al., 2005; Fraser and Bickmore, 2007).

Pk-1 Expression in Chicken Embryos

Whole-mount in situ hybridization (WISH) was used to examine the expression of *pk-1* and *pk-2* transcripts in prestreak and Hamburger and Hamilton stage (HH) 3–HH20 chicken embryos. Probe-specific expression of *pk-1* mRNA was observed in the area opaca of prestreak embryos (Fig. 2A) and in the primitive streak of HH3 chick embryos (Fig. 2B). Sections of HH3 embryos confirmed the presence of pk-1 transcripts in the streak and overlying epiblast (Fig. 2B′). Expression continued in the primitive streak from HH4–HH10 (Fig. 2C,C′,C″,D,D″,E,E‴,F) and was detected in the posterior part of Hensen's node at HH4 (Fig. 2C,C′) and Hensen's node from stage HH5 to HH10 (Fig. 2D,E,E‴,F,J). From HH5, expression was detected in the midline of the neural plate (floor plate) and the underlying head process (notochord; Fig. 2D,D′,E,E″). At HH10, expression was still present in the floor plate and underlying notochord, particularly in the hindbrain region and the anterior neural tube (Fig. 2F,G). Expression was also detected in the foregut endoderm at HH8 and HH10 (Fig. 2E′,G). Weak expression was observed in the somitocoel (Fig. 2H). In caudal regions of the embryo, the neural plate, Hensen's node, and the adjacent paraxial and lateral plate mesoderm expressed high levels of *pk-1* mRNA (Fig. 2F,I,J). At HH17, *pk-1* mRNA was expressed in emerging limb buds, and in somites (Fig. 2K–O). In epithelial somites, *pk-1* mRNA was enriched dorsally (Fig. 2N,O), and in differentiating somites, *pk-1* transcripts became restricted to the dorsomedial lip of the dermomyotome (Fig. 2L,M). The dorsomedial lip has been characterized as a morphogenetic center, through which epaxial myotome precursors migrate from the dermomyotome by direct ingression and bidirectional extension (Gros et al., 2004). It is therefore interesting to note that the somitic expression of *pk-1* was strikingly similar to chicken *flamingo-1*, another PCP signaling component (Formstone and Mason, 2005). Although some nonspecific trapping of dye was detected in head tissues, specific signal for *pk-1* transcripts was robustly detected in a striped pattern across the trunk with strong expression in the limb buds at HH20 (Fig. 2P). Sections revealed that *pk-1* was expressed in the epaxial and hypaxial myotomes and throughout the limb bud mesenchyme and lateral body folds (Fig. 2Q). Furthermore, *pk-1* was expressed in the ventral metencephalon and in tissue surrounding the aorta (Fig. 2R), similar to zebrafish (Carreira-Barbosa et al., 2003).

Pk-2 Expression in Chicken Embryos

Chicken *pk-2* transcripts were first detected at HH4 in the developing neural plate and Hensen's node (Fig. 3B,B′,B″). Expression continued from HH5 to HH7 in Hensen's node and in neural ectoderm where it was restricted to the midline (Fig. 3C,C′,C″,D,D′,D″). At HH11, robust *pk-2* expression was seen in somites (Fig. 3E,G,H) and weak *pk-2* mRNA levels were detected in the floor plate of the neural tube in later embryos (HH11; Fig. 3F). In somites, *pk-2* mRNA was observed in the ventromedial domain of epithelial somites, where cells undergo an epithelial to mesenchymal transition to form the sclerotome (Fig. 3G,H). In epithelial somites, *pk-1* and *pk-2* mRNAs were localized in nonoverlapping domains (Figs. 2N,O, 3H). *pk-2* mRNA was absent in the most recently formed somite pair (Fig. 3I). At later stages, *pk-2* expression was observed in recently formed somites, but not in the youngest somite pair (Fig. 3J). At these older stages, some nonspecific staining was observed in the head tissues due to trapping of the substrates (Fig. 3J,K). At HH20, *pk-2* was expressed in distal limb bud mesenchyme and the most recently formed somites in the tail bud (Fig. 3K–O).

The expression of chicken prickle genes is similar to the expression described in pregastrulation and gastrulation stage mouse embyros (Crompton et al., 2007). Both mouse and chick *pk-1* were expressed in the primitive streak and mesoderm tissues. However, in addition, *pk-1* was expressed in the early neural plate. During gastrulation, *pk-2* was expressed in the node and Hensen's node in mouse and chick, respectively. The striking, mutually exclusive expression of vertebrate prickle homologues in dorsal and ventral regions of epithelial somites, in differentiating myoblasts of the myotome (*pk-1*), and in the mesenchyme of developing limb buds (*pk-1* and *pk-2*) is described here for the first time. Of interest, PCP signaling has not previously been implicated in epithelial somites or limb bud mesenchyme. In contrast, both *pk-1* and *pk-2* show overlapping expression with components of canonical Wnt signaling, which have been shown to be expressed in the primitive streak (*Tcf-1*, β-*catenin* and *Lef-1*), the neural plate (*Tcf-3*), the dorsomedial epithelial somites and myotome (β-*catenin* and *Lef-1*), and the limb bud mesenchyme (β-*catenin* and *Tcf-3*; Schmidt et al., 2004). It will therefore be interesting to characterize further the importance of vertebrate prickle genes in PCP signaling, as antagonists of canonical Wnt signaling (Veeman et al., 2003; Chan et al., 2006), as well as in other alternative signaling pathways, such as the Notch pathway.

EXPERIMENTAL PROCEDURES

Cloning of Chicken Prickle Homologues

Chicken *pk-1* and *pk-2* fragments were amplified by PCR from cDNA prepared from stage 8–14 chicken embryos using standard molecular biology protocols (Münsterberg and Lassar, 1995). Primers were designed using predicted *prickle* sequences derived from the Ensembl Genome Browser (www.ensembl.org). See Table 1 for accession numbers. *Prickle-1* primer sequences were

prickle-1-*Bam*HI (forward, 5′-GGATCCATGGAGCCCAAAGCTAAC-3′) and prickle-1-*Not*I (reverse, 5′-GCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAAGAAATTATGCAATTTTTC-3′), including hemagglutinin tag. *Prickle-2* primer sequences were prickle-2 *Xba*I (forward, 5′-TCTAGAATGCCCCTGGAGATGGAG-3′) and prickle-2-*Not*I (reverse, 5′GCGGCCGCTCAAGCGTAATCTGGAACATCGTATGGGTAGGATATGATACAGTTTG-3′), including hemagglutinin tag. PCR products were cloned into pGEM-T (Promega) and sequenced. The accession numbers for protein and cDNA sequences for various species were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html).

WISH and Paraffin Sections

Fertilized eggs were incubated at 37°C until the desired stage of development was reached (Hamburger and Hamilton, 1951). The embryos were collected into DEPC-treated phosphate buffered saline, fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through ascending grades of PTW/methanol washes, and stored in methanol at −20°C. Antisense digoxigenin-labeled riboprobes corresponding to the full-length coding sequence for chicken *pk-1* and *pk-2* were synthesized using linearized pGEM-T plasmid using T7 and SP6 RNA polymerases (Promega), and WISH was performed as previously described (Smith et al., 2005). After in situ hybridization, the embryos were fixed in 4% paraformaldehyde overnight and photographed. For paraffin sectioning, embryos were dehydrated through ascending grades of ethanol and cleared in xylene. The embryos were incubated in paraffin wax at 65°C overnight and embedded, and 10-μm sections were cut, collected on TESPA-coated slides, dewaxed, and coverslipped with Entellan (Merck, Germany).

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