Expression of Hex during feather bud development

AKIKO OBINATA*1 and YOSHIHIRO AKIMOTO2

1Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa, Japan and 2Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo, Japan

ABSTRACT We studied proline-rich divergent homeobox gene Hex/Prh expression in the dorsal skin of chick embryo during feather bud development. Hex mRNA expression was first observed in the dorsolateral ectoderm and mesenchyme at 5 days, then in the epithelium and the dermis of the dorsal skin before placode (primordium of feather bud) formation and then was restricted to the placode and the dermis under the placode. Afterward, Hex expression was seen in the epidermis and the dermis of the posterior region of short bud. In accordance with Hex mRNA expression in the placode, Hex protein was observed in the epidermis as well as in the dermis of the placode. Immunoelectron microscopic study indicated that the protein located both in the nuclei and cytoplasm of the epidermis and the dermis at the short bud stage. The Wnt signaling pathway plays an essential role in the early inductive events in hair (Wnt3a and 7a) and feather (Wnt7a) follicles. The pattern of Hex expression in the epidermis was similar to that of Wnt7a, while little, if any, expression of Wnt7a was detected in the dermis under the placode or the dermis of the short bud compared with that of Hex, suggesting that Hex plays an important role in the initiation of feather morphogenesis.

KEY WORDS: Hex, homeobox gene, feather placode, Wnt, feather bud development

Epithelial appendages including feathers, scales, hair, claws, teeth, etc are induced and shaped through epithelial-mesenchymal interactions (Smola et al., 1993; Chuong et al., 1996; Kishimoto et al., 2000). An inductive signal from the dermis initiates formation of epidermal placodes that, in turn, induce dermal condensation in the underlying dermis (reviewed in Sengel, 1976). Several molecules that mediate inductive signaling during hair and feather tract formation have been identified, including Wnts (Widelitz et al., 1999; Noramly et al., 1999; Huelsken et al., 2001; Andl et al., 2002), bone morphogenetic protein (BMP) in early skin development (Scaal et al., 2002), BMP inhibitor at placode stages (Patel et al., 1999), fibroblast growth factors (FGFs)(Widelitz et al., 1996; Song et al., 1996), Hedgehog (Ting-Berreth and Chuong, 1996) and Notch/Delta families (Crowe et al., 1998; Viallet et al., 1998). Notch/Delta signals refine the patterning of the feather placode (Crowe et al., 1998).

Homeobox genes are a large family of transcription factors which play a fundamental role in cell differentiation during development (Gehring et al., 1994). Abnormal hair follicles were observed in transgenic mice overexpressing homeobox gene Msx-2 (Jiang et al., 1999). Hair defects were observed in Hoxc13 mutant mice (Godwin and Capece, 1998) and Jave-Suarez et al., (2002) showed direct involvement of HOXC13 in the regulation of human hair keratin gene expression. The divergent homeobox genes Msx1 (Noveen et al., 1995), Gbx1 (Obinata et al., 2001) and HB9 (Kosaka et al., 2000a,b) are expressed in skin and its appendages, such as hair, feather or scale and appear to be candidates for the regulation of the development of these tissues. Another divergent homeobox gene Hex is expressed during early stages of chick embryogenesis, including pharyngeal endoderm, endocardium, liver, thyroid gland primordia and blood islands (Yatskievych et al., 1999). Hex is required for forebrain, thyroid and liver formation and blood differentiation (Keng et al., 2000; Martinez-Barbera et al., 2000; Martinez-Barbera & Beddington, 2001). In liver morphogenesis, Hex expression in avian anterior lateral endoderm is regulated by autocrine BMP signaling (Zhang et al., 2002). We showed previously that Hex is expressed in chick embryonic tarsometatarsal skin and regulates epidermal cell proliferation (Obinata et al., 2002). In this study, to examine whether the Hex gene is involved in the feather morphogenesis or not, we performed in situ hybridization and immunostaining analyses spatially and temporally in dorsal skin of chick embryo.

Expression pattern of Hex during feather bud development

To see the localization of Hex during feather bud development, we performed an in situ hybridization analysis in developing chick. Hex expression was seen in the dorsolateral mesenchyme...
Fig. 1. Expression pattern of *Hex* mRNA during feather development as revealed by *in situ* hybridization. Transverse sections (A,B,D) through the dorsal ectoderm and trunk region of chick embryos and whole embryo (C,E,H) after whole mount *in situ* hybridization (WISH) (A-E, H). *Hex* expression is seen in the dorsolateral region (arrows) of the mesenchyme, if any, (A,C) and is strong in liver (L) (B) at 4 days. The expression is seen from the dorsolateral to dorsomedial region (arrows) of the ectoderm and mesenchyme (D,E) at 5 days. Cryostat sections (F,G,I-M). *Hex* expression is seen through the epidermis (Ep) while the expression was very little in the dermis (De) of the dorsal skin before placode formation (F) at 6 days and is restricted to the epidermis and dermis of the placode region (arrows) (G) at 7 days. Later, at short bud stage, the stronger expression is seen in both the epidermis and the dermis at the posterior buds (I,J). At long bud stage, the signal is stronger in the epidermis than in the dermal cells in the posterior bud (K-M). In WISH (H), as well as in cryostat sections, *Hex* gene expression is detectable in feather buds with stronger signal in posterior regions, but not in interbud regions in 8-day-old chick embryo. a, anterior; p, posterior. Bars, 100 µm in A,D,F,G,I-M; 500 µm in B; 1 mm in C,E; 2 mm in H.
Hex expression in feather bud

Hex expression was seen through the epidermis of the dorsal skin before placode formation at 6 days while the expression was very little in the dermis (Fig. 1 F) and is restricted to the placode epithelium and the dermis underneath the placode at 7 days (Fig. 1G). Later, at short bud stage, stronger Hex expression was seen in both the epidermis and the dermis of the posterior bud (Fig. 1 H, I, J). At long bud stage, the signal was stronger in the epidermis than in the dermis at the distal region of the bud (Fig. 1 K, L) and later an intense signal in the dermis at the bottom region of the bud adjacent to the epidermis was also seen (Fig. 1 M). Surprisingly, the Wnt7a expression pattern in the epithelium (Fig. 2 A, B, C and Widelitz et al., 1999) was almost similar to that of Hex (Fig. 1 F, G, I), while less expression of Wnt7a in the dermis relative to that of the epidermis was observed under the placode (compare Fig. 1G with Fig. 2C).

Immunohistochemical localization of Hex protein during feather bud development

To know whether Hex mRNA and Hex protein are expressed correlatively and Hex locates in nucleus as a transcription factor during feather bud development, we used two different kinds of antiserum. One is antiserum raised against glutathione S-transferase fusion protein containing the 76 COOH-terminal amino acids of mouse Hex (Ghosh et al., 2000), which was

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**Fig. 2. Expression pattern of Wnt7a mRNA during feather development as revealed by in situ hybridization.** Sections of dorsal skin from 6-8-day-old chick embryos (A-C). Wnt7a expression is seen through the epidermis before placode formation (A) at 6 days and the stronger signal is seen in the epidermis of the placode region (B) at 7 days. At late placode stage, the stronger signal is seen in the epidermis of the posterior buds (C). Bar, 50 μm.

**Fig. 3. Western blot and immunohistochemical staining of Hex protein during feather development.** Whole-cell lysates (50 μg) obtained from HeLa cells transfected with Hex-pcDLSRx (A) and chick embryonic tissues (B). See text for details. Numbers used at the left side indicate molecular weights. Hex protein is detected by anti-N-terminal chick Hex antiserum followed by Cy3-conjugated secondary antibody (C-F). Hex was observed through the epidermis and the dermis with a stronger immunoreactivity at the placode (C) and the short bud (D, E), while less immunoreactivity was observed in the dermis (C, D, E). At the long bud stage, the immunoreactivity was seen throughout the epidermis and the periderm at the bud with a stronger immunoreactivity in the peridermal cells and the dermis at the root regions of the bud (F). Bar, 50 μm.
Fig. 4. Immunoelectron microscopic study of feather bud at 10 days. Anti-N-terminal chick Hex antiserum (A-D) and antibody against glutathione S-transferase fusion protein containing the 76 COOH-terminal amino acids of mouse Hex (E-H) were used. Colloidal gold labeling represents the localization of Hex in the epidermal cells of superficial layer (A,E), intermediate layer (B,F), basal layer (C,G) and in the dermal fibroblasts (D,H). Both antibodies indicated the same localization pattern in nucleus and cytoplasm of the epidermis and the dermis in the bud at 10 days with stronger immunoreactivity in the cytoplasm (A-H). BC, basal cell of epidermis; BL, basal lamina; D, dermis; N, nucleus. Bar, 1 µm.

that of its mRNA, the extent of their expression level was different. The periderm is sloughed at later stages in the development. To study more precisely about the localization of the Hex in the cell, immunoelectron microscopic study was performed with 2 different kinds of Hex antibody. Colloidal gold labeling represents the localization of Hex in the epidermal cells of superficial layer (Fig. 4 A,E), intermediate layer (Fig. 4 B,F), basal layer (Fig. 4 C,G) and in the dermal fibroblasts (Fig. 4 D,H) of the long bud at 10 days. Both antibodies indicated the same localization pattern in nucleus and cytoplasm of the epidermis and the dermis with stronger immunoreactivity in the cytoplasm (Fig. 4 A-H). Few colloidal gold labeling was observed in the skin without first antibody (Fig. 5).

In this study, we showed that Hex was expressed as early as at 5 days in chick embryonic dorsolateral skin and then diffusely distributed in skin before placode formation and that its expression was restricted to the placode and the dermis under the placode and later in the epidermis and the dermis of the posterior region of the short bud. The Hex protein localized both in nucleus and cytoplasm of the epidermis and the dermis at the long bud stage with stronger expression in the cytoplasm, suggesting Hex might be actively sequestered in the cytoplasm, either by preventing nuclear import or by promoting a balance of export over import signals, or have a potential for intercellular trafficking by conventional secretion as in the case of Engrailed homeoprotein (Maizel et al. 2002) or CVC paired-like homeobox proteins (Knauer et al., 2005). The meaning of Hex existence in the cytoplasm must be studied in future. Surprisingly, the pattern of Hex expression in the epidermis was similar spatially and temporally to that of Wnt7a expression. The Wnt/Wg signaling pathway reviewed in Wodarz & Nusse, (1998) plays an essential role in the early inductive events in hair and feather follicles (Widelitz et al., 1999; Huelsken et al., 2001; Andl et al., 2002) and even in its equivalent (dentine) of fly (Dai et al., 1998; Payre et al., 1999; Li et al., 2002) and in many aspects of development such as the tooth (Dassule & McMahon, 1998), limb bud (Cygan et al., 1997), lung (Shu et al., 2002), mammary gland (Humphreys et al., 1997),

kindly provided by Dr C. Bogue. The other is anti-N-terminal chick Hex antiserum. By Western blots, they developed a specific strong immune response against a 41k dalton band of Hex recombinant protein (Fig. 3A), the homogenate of chick embryonic dorsal skin, intestine, liver (Fig. 3B), or 5-day-old whole embryo (data not shown). At placode stage and later, Hex was observed throughout the epidermis with a stronger expression at the placode and bud region and in the dermis under these regions (Fig. 3 C-E). At the long bud stage, the immunoreactivity was seen at the bud with a stronger immunoreactivity in the peridermal cells and the dermal cells at the root regions of the bud (Fig. 3F). It is interesting to note that, while tissue localization of Hex protein was correlated with
Kidney (Torres & Nelson 2000) and liver (Suksawang et al., 2004). Recent studies have now addressed the issue of whether Hex is involved in the Wnt7a signaling pathway or in the initiation of feather bud formation (Obinata and Akimoto, 2005).

**Experimental Procedures**

**Preparation of a digoxigenin (DIG)-labeled RNA probe**

The Hex RNA probe was prepared as described previously (Obinata et al., 2002). For synthesis of a Wnt7a RNA probe, a Wnt7a cDNA fragment containing the entire coding region, which was kindly provided by Dr T. Nohno (Kawakami et al., 2000), was amplified and prepared using a standard protocol.

**In situ hybridization.**

In situ hybridization with the DIG-labeled probe was performed as described previously (Kosaka et al., 2000a).

**Transgene construction**

A full-length Hex cDNA containing the entire Hex coding region, which was generously provided by Dr G. Goodwin (Haddow Laboratories, Institute of Cancer Research, Sutton, UK), was constructed with pcDLRα.

**Lipofection**

HeLa cells were transfected with Hex -pcDLRα using TransIT-LT1 Transfection Reagent (Invitrogen, California, USA) according to the manufacturer’s instructions.

**Western blotting**

A rabbit antiserum was generated against a KLH-conjugated chick Hex NH2 peptide (MOYOQAPGAAAPAAALC). Western blotting was performed using standard protocols.

**Immunostaining for light and electron microscopy**

Frozen skin sections were processed for immunohistochemical staining as described previously (Akimoto et al., 1992; Kosaka et al., 2000b).

**Microscopy**

Skin explants were processed for light and electron microscopic observations as described previously (Obinata et al., 1991).

Acknowledgements

We thank Dr G. Goodwin (Haddow Laboratories, Institute of Cancer Research, Sutton, UK), for providing a chick Hex cDNA; Dr C. Bogue (Department of Pediatrics, Yale University, School of Medicine, New Haven, Connecticut) for providing a Hex polyclonal antibody and Dr T. Nohno (Department of Molecular Biology, Kawasaki Medical School, Kurashiki, Japan) for providing chick Wnt7a cDNA. We are grateful to Ms. S. Matsubara and Ms. T. Shibata for their technical support. This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Science, Sports, Culture and Technology, Japan.

References


