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Molecular cloning of chicken *Cecr2* and its expression during chicken embryo development

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ABSTRACT Cecr2 is a transcription factor involved in neurulation and chromatin remodeling. In the present study, the full length of the coding sequence of the chicken orthologue *Cecr2* was obtained by RT-PCR. Sequence analysis and alignment showed that it contained an AT hook, as well as a bromodomain which was highly conserved among different species, consistent with its role in chromatin remodeling. The expression pattern of chicken *Cecr2* was subsequently investigated during the development of the chicken embryo by *in situ* hybridization. In addition to its predominant expression in neural tissues during neurulation, *Cecr2* was also found to be expressed in the developing somites and in the intermediate zone of the spinal cord, suggesting that it may play a role in somite and neuronal development.

KEY WORDS: *Cecr2, myotome, spinal cord, neurulation, chicken embryo*

Introduction

Cecr2 was first identified in the region q11.2 of human chromosome 22 (Footz *et al.*, 2001). The full length of the coding region is 4392bp, containing an AT hook and a bromodomain that are present in many chromatin remodeling proteins. The protein product CECR2, together with SNF2L, is involved in chromatin remodeling *in vitro* (Banting *et al.*, 2005). In the mouse, *Cecr2* transcripts are mainly expressed in neural tissues such as neural folds, recently closed neural tube and limb mesenchyme. Mutation of *Cecr2* leads to a high rate of exencephaly and prenatal death (Banting *et al.*, 2005), indicating that *Cecr2* is involved in neurulation.

In the chicken, three ESTs of *Cecr2* are reported in BBSRC ChickEST Database (http://www.chick.manchester.ac.uk/). In our study, we cloned the full length of the coding sequence of chicken *Cecr2* and investigated its expression pattern during the development of the chicken embryo. In addition to the expression pattern which resembles that of the neurulation stage in the mouse embryo, the signal present in the somite and the spinal cord argued for potential functions of *Cecr2* during embryo development, in addition to its roles reported.

Results

Molecular cloning and sequence analysis

The coding sequence of chicken *Cecr2* has a length of 4422bp (Accession No: EU850803), encoding a putative protein of 1473 amino acids (Fig. 1). Motif discovery with Pfam revealed that the protein contains a bromodomain and an AT hook (Fig. 1). Sequence alignments showed these two motifs are highly conserved among human, mouse, chicken and zebrafish Cecr2 (Fig. 1).

Expression of Cecr2 *during chicken embryo development*

Neural tissues

Cecr2 transcripts were present in the neural folds and newly formed neural tube from HH stage 8 onwards (Fig. 2 A,B,D,E). As the neural tube matured and partitioned rostrocaudally, the expression of *Cecr2* was gradually restricted to the dorsal region of the neural tube and brain vesicles (Fig. 2 C,F,G,H). After HH stage 19, the signal extended towards the ventral side of the intermedi-

Abbreviations used in this paper: Cecr2, cat eye syndrome chromosome region, candidate 2; DML, dorsal medial lip; DRG, dorsal root ganglia; VLL, ventral lateral lip.

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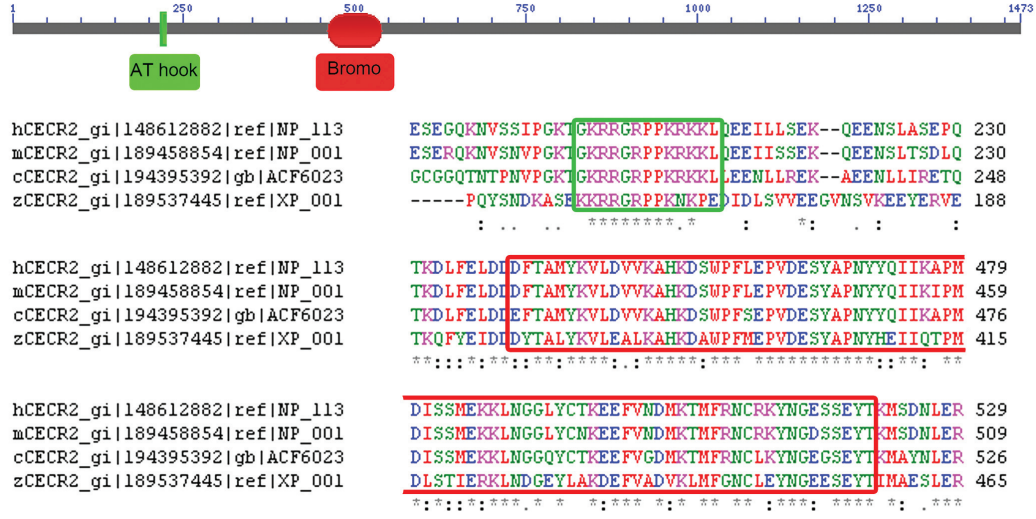
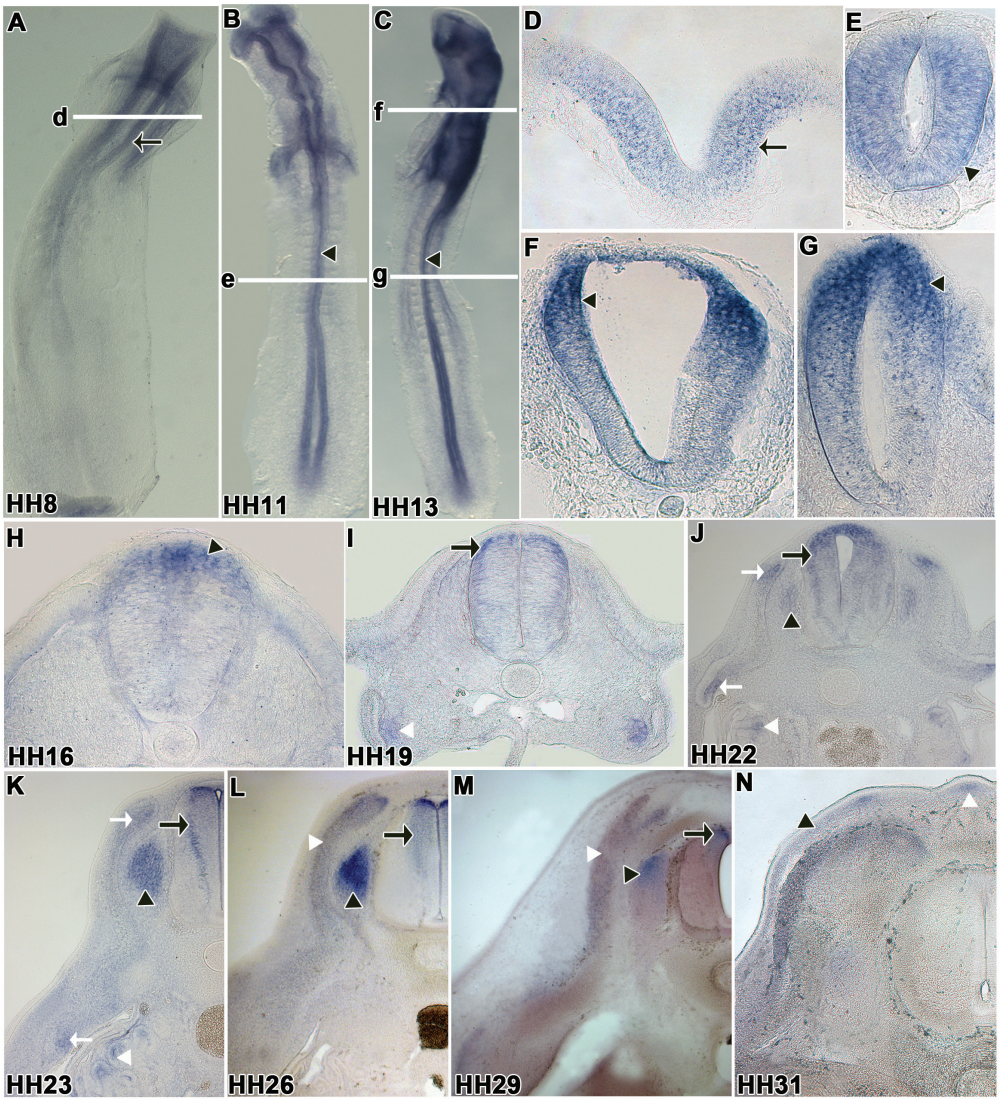


Fig. 1. Cecr2 sequence analysis and alignments. *The approximate positions of AT hook and bromodomain in the protein are marked on the scaled line representing the full length of cCecr2. In sequence alignment, green box outlines the AT hook. Red line delimits the bromodomain.*



ate layer of the spinal cord and was maintained there (Fig. 2 I,J,K). After HH stage 26, the expression in the intermediate layer declined (Fig. 2 L,M) until completely vanishing at HH stage 31 (Fig. 2N). Notably, *Cecr2* was expressed in the dorsal root ganglia (DRG) from HH stage 22 to 29 as well (Fig. 2 J,K,L,M).

Somites and limb buds

At HH stage 16, the expression of *Cecr2* was observed in the mesenchyme of the lateral plate-derived mesoderm at limb level (Fig. 3 A,B). The expression domain in the mesenchyme increased as the limb bud grew (Fig. 3D). From HH stage 18, *Cecr2* appeared at the cranial and caudal border of rostral somites (Fig. 3 C,D). At around HH stage 22 or 23, the dorsal medial and ventral lateral regions of the somites were positive for *Cecr2* expression (Fig. 3 E,F). Sections revealed that the expression was located in the sub-lips of the dorsal medial lip (DML) and ventral lateral lip (VLL) (Fig. 2 J,K). During these stages, the signal in limb buds was gradually restricted to the distal mesenchyme (Fig. 3 E,F). It completely vanished in the mesenchyme, but was present in the dorsal and ventral myogenic zones after HH stage 27(Fig. 3K), while in the somite, it was further

Fig. 2. *Cecr2* expression in neural tissues and somites. (A,B,D,E) *Expression is present in neural folds (black arrows) and newly formed neural tube (black arrowheads) from HH stage 8 to 11.* **(C,F,G,H)** *The signal is restricted to the dorsal region of the neural tube and brain vesicles from HH stage 13 to 16 (black arrowheads).* **(H-N)** *The expression in the intermediate layer of the spinal cord (black arrows) and DRG (black arrowheads) is dynamic, appearing from HH stage 19 and disappearing at HH stage 31. In somites,* Cecr2 *is expressed in sub-lips (white arrows in* J,K*) of DML and VLL, mesenchyme (white arrowheads in* L,M*) originating from dermomyotome, epidermis (black arrowhead in* N*) and feather buds (white arrowhead in* N*). The signal is also positive in the mesonephric duct (white arrowheads in* I,J,K*).*

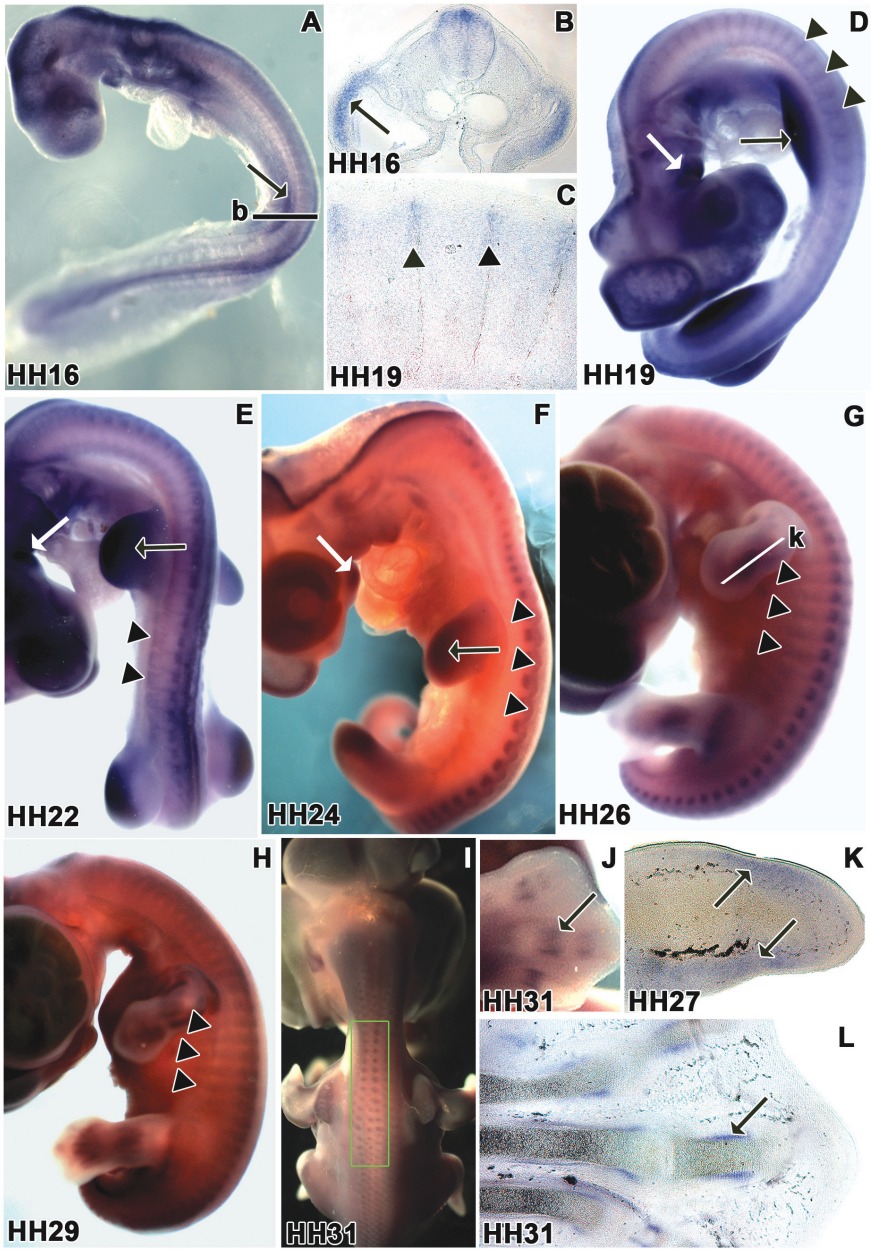


Fig. 3. Expression of *Cecr2* in limb buds and somites. *In limb buds,* Cecr2 *transcripts are strongly expressed in the mesenchyme (black arrows in* **A,B,D***), and gradually restricted to the distal region (black arrows in* **E,F***). They are present in the dorsal and ventral myogenic zone (black arrows in* **K***, which comes from cross section of G at forelimb level) and perichodrium (black arrows in* **J,L***) of developing digits.* **(D-H)** *Overview of* Cecr2 *expression in somites from the whole mount* in situ *hybridization, which is further revealed in detail by sections illustrated in Fig. 2. In somites,* Cecr2 *is initially expressed in the rostral and caudal borders of somites (black arrowheads in* **C**,**D***. C shows sagittal section of D). The transcripts later are present in the ventral lateral region (black arrowheads in* **E***) and dorsal medial region (black arrowheads in* **F***) of the somites. The expression becomes moderately positive through the somites (black arrowheads in* **G,H***). The expression is also positive in pharyngeal arches (white arrows in* **D,E,F***). At HH stage 31, the signal is present in feather buds (green frame in* **I***).*

restricted to the mesenchyme originating from the dermomyotome and DML(Fig. 2 L,M). At HH stage 31, the transcripts were observed in feather buds (Fig. 2N, Fig. 3I), dermis (Fig. 2N) and perichondrium of the developing digits (Fig. 3 J,L) as well.

Other tissues

Cecr2 expression was also observed in the mesonephric duct (Fig. 2 I,J,K) and pharyngeal arches (Fig. 3 D,E,F).

Comparisons of expression pattern between* Cecr2 *and* Frek *in somites

From HH stage 19 to HH stage 21, both *Cecr2* (Fig. 4 A,B) and *Frek* (Fig. 4 E,F) appeared at the rostral and caudal borders of somites at interlimb level. Sections revealed that *Cecr2* occupied the dorsal region of the myotome (Fig. 4 a,b), while *Frek* was expressed in the ventral region of the myotome (Fig. 4 e,f). From HH stage 23 to HH stage 25, *Cecr2* expression was restricted to the dorsal medial and ventral lateral regions of somites (Fig. 4 C,D). Sections revealed that the expression was located in the sub-lips of DML and VLL (Fig. 4 c,d). In contrast, *Frek* extended through the whole somite (Fig. 4 G,H). Sections showed that it was expressed in the whole myotome (Fig. 4 g,h).

Discussion

Roles of conserved domains in Cecr2

The bromodomain can interact specifically with acetylated lysine (Haynes *et al.*, 1992), and may be involved in protein-protein interactions as well as the assembly or activity of multi-component complexes involved in transcriptional activation (Tamkun, 1995). It is 110 amino acid long and found in many chromatin-associated proteins. The so-called AT hook is a DNA binding motif with a preference for A/T rich regions. It is suggested that proteins with AT hook could function in nucleosome phasing (Reeves and Nissen, 1990). Both of the two motifs are characteristic of many chromatin remodeling proteins (Flaus and Owen-Hughes, 2001). *In vitro* study reveals that Cecr2 is indeed involved in chromatin remodeling (Banting *et al.*, 2005). The high conservancy of these two motifs among different species suggests that Cecr2 may function in the same conserved chromatin-remodeling way among different organisms.

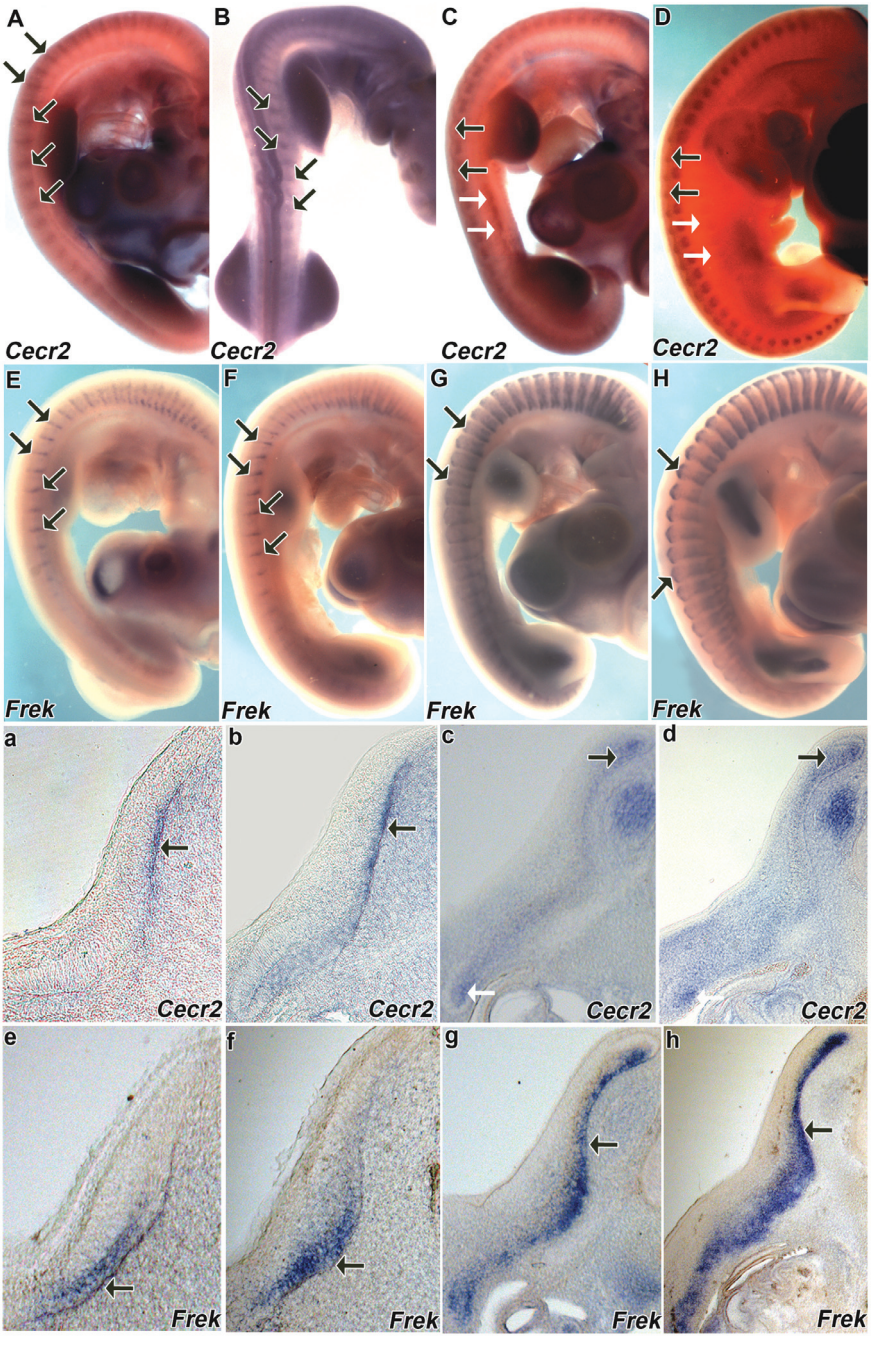
Possible roles of* Cecr2 *during embryo development

Myotome development

Myotome development is a multi-step process that comprises four waves of cell recruitment. The first wave arises from the dorsomedial wall of the epithelial somite where cells spread underneath the dermomyotome as a scaffold for the second

wave of postmitotic cells which arise from all four edges of the dermomyotome (Gros *et al.*, 2004; Kahane *et al.*, 1998a; Kahane *et al.*, 1998b). The third wave cells enter from the rostral and caudal lips of the dermomyotome. They are mitotically active, and express *Frek* (Kahane *et al.*, 2001). Finally, cells delaminate from the center of the dermomyotome to enter the myotome, making up the fourth wave of mitotically active, *Frek*-expressing muscle precursors (Gros *et al.*, 2005).

Of the four waves that contribute to myotome formation, two populations of cells have origins from rostral and caudal lips of the dermomyotome. One population belongs to the third wave of which cells are *Frek* positive (Kahane *et al.*, 2001). The other one belongs to the second wave of which cells arise from four borders of the dermomyotome (Gros *et al.*, 2004; Kahane *et al.*, 1998a). In our observation, though *Cecr2* is expressed in the rostral and caudal borders of the somites, it has a distinctly different expression pattern from *Frek*. In brief, *Cecr2* expression is restricted to the four borders of the dermomyotome, while *Frek* extends through the whole myotome from the rostral and caudal lips. The disparity distinguishes the *Cecr2*-expressing cells from the *Frek*-positive cells, thus ruling out the possibility of *Cecr2* expressed in cells of the third wave. Additionally, *Cecr2* expression is concomitant with the initial phase of cell dynamics of the second wave. Accordingly, we suggest that *Cecr2* is expressed in subpopulation of the second-wave cells during myotome development, probably the ones arising from the four edges which correlate with *Cecr2* expression.



Neural development

A study in the mouse shows that *Cecr2* is expressed in the neural folds and the recently closed neural tube and that mutation of mouse *Cecr2* leads to exencephaly. These results indicate that *Cecr2* is involved in neurulation (Banting *et al.*, 2005). In support, there is a similar expression pattern during neurulation in the chick embryo. After neurulation, the *Cecr2* expression domain is gradually restricted to the dorsal neural tube. During this period, the ventral signal Sonic hedgehog (SHH) and the dorsal signal of Tgf-β proteins are shaping the dorsal-ventral polarity of the neural tube (Wilson and Maden, 2005). The recession of *Cecr2* expression may be related to such signaling networks. Continuous expression of *Cecr2* is also observed in the intermediate layer of the spinal cord and DRG, which consist of postmitotic neuronal cells imminent to further differentiate (Lee *et al.*, 1995; Roztocil *et al.*, 1997). Persistent expression of *Cecr2* in these two regions suggests that *Cecr2* may function during neuronal development.

Fig. 4. Expression of *Cecr2* and *Frek* in somites. **(A,B)** Cecr2 *is expressed in the rostral and caudal borders of somites (black arrows) at HH stage 19 and HH stage 21.* **(a,b)** *Cross sections from (A,B) show* Cecr2 *expression in the dorsal region of the cranial and caudal borders of the somite at interlimb level (black arrows).* **(C,D)** *The expression of* Cecr2 *is moderately positive in the dorsal medial region (black arrows) and ventral lateral region (white arrows) of somites at HH stage 23 and HH stage 25. Sections reveal that the expression is located in the sub-lips (black and white arrows in* **c,d**) *of DML and VLL.* **(E,F)** *Expression of* Frek *in the rostral and caudal borders (black arrows) of somites at HH stage 19 and HH stage 21.* **(e,f)** *Cross sections from E, F show* Frek *expression in the ventral region of the cranial and caudal borders of the somite at interlimb level.* **(G,H)** *The expression of* Frek *extends through somites.* **(g,h)** *Cross sections from G, H indicate that* Frek *transcripts are positive through the whole myotome (black arrows).*

TABLE 1

PRIMERS FOR RT-PCR OF CHICKEN *CECR2*

	Sense primers	Antisense primers
1	AGATGTGCCCGGAGGAAG	CTCTGCGCCTCTGCTTCTCCATTG
2	CCATCAGCCCATCAAACCAATCAA	ATCCGTGTCTCCATCTTCCCC
3	ACACGGATGAGGAGTTCTGGATCA	CGGCCCCATAGGTGCCTTCTCCTC
4	ACCGGCCCTACAAGTACCTGAATC	GGCGGCTGGCGAGGGTGGTAAG
5	ATCAGACCACCTGGGAAAAC	GGCATGTAGAGAGGGTGGAA
6	GCTGCTAGCCTGATGGAGAAGCCCCTC	CGCTTGGAGAAACCTCTTTG
7	CGTAGCGAGTGCCAAAGAG	TGAGGTCCTTCCTTCCTCAG

Limb development

Cecr2 transcripts persist in the mesenchyme of the limb bud from HH stage 16 to 21, but gradually diminish along the proximal-distal (P-D) axis afterwards. Fgf10 has the same expression pattern in the limb bud. It interacts with Fgf8 from the apical ectodermal ridge (AER) to maintain the outgrowth of the limb bud (Ohuchi *et al*., 1997). Possibly, *Cecr2* may correlate with *Fgf10* or its signaling cascade during early limb development. Besides, presence of *Cecr2* in the perichondrium of developing digits implies its participation in the chondrogenic processes as well.

Materials and Methods

RT-PCR and sequence analysis

Total chicken mRNA was extracted with Trizol reagent (Invitrogen). Seven pairs of primers were used for RT-PCR (Table 1). The amplified fragments were purified from 1% agarose gel. cDNA fragments of interest were cloned into the pDrive vector (Qiagen) and then sequenced. Sequence analysis was performed with Pfam (Finn *et al*., 2006). Sequence alignments were made with clustalW2 provided in EBI (http://www.ebi.ac.uk/Tools/clustalw2/).

Probe preparation

A 967 bp cDNA fragment of chicken *Cecr2* was obtained by RT-PCR. The fragment was ligated into the pDrive vector. The pDrive-*Cecr2* plasmid was linearised with *HindIII*, and the antisense probe was synthesized with T7 RNA polymerase. The sense probe as control was synthesized with SP6 RNA polymerase after linearising the plasmid with *BamHI*. *Frek* probe was prepared from plasmid presented by Marcelle and colleagues (Marcelle *et al*., 1994).

In situ *hybridization and sectioning*

Fertilized chicken eggs obtained from a local breeder were incubated at 38ºC, 80% humidity. Embryos were staged according to Hamburger and Hamilton (1951), sacrificed and fixed in 4% PFA/PBT. Whole mount *in situ* hybridization was performed as described (Nieto, 1996). Embryos were sectioned at a thickness of 40–60µm using a Leica vibratome. Sections were photographed with Leica DFC320 digital camera mounted to the microscope Axioscope 20 from Zeiss.

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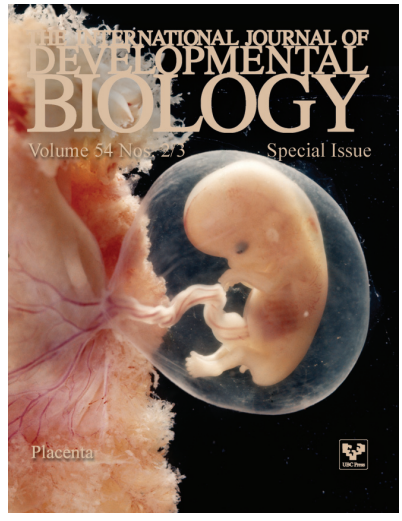

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