

Isolation and characterization of chicken β -catenin

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Abstract

β -catenin interacts with a number of proteins in different important biological processes, including cell adhesion through cadherins, actin organization through fascin, body axis determination through Wnt signaling, tumor suppression through APC, and transcriptional activation through LEF-1. To examine its function in chicken embryogenesis, we isolated the chicken homolog of β -catenin from a chicken embryo cDNA library. The sequence is highly conserved at the amino acid level between chicken, mouse (99%), human (99%) and *Xenopus* (97%). In-situ hybridization and immunostaining showed that in the developing limb, it is specifically expressed in the apical ectodermal ridge, suggesting a role in epithelial–mesenchymal interactions. © 1997 Elsevier Science B.V.

Keywords: cDNA cloning; In-situ hybridization; Limb; Apical ectodermal ridge

1. Introduction

β -catenin is a vertebrate homolog of the *Drosophila* segment polarity gene product Armadillo (McCrea et al., 1991; Butz et al., 1992). It is involved in establishing the body axis. Injection of antibodies against β -catenin or of synthetic mRNA encoding β -catenin into the ventral side induced a duplication of the body axis (McCrea et al., 1993; Funayama et al., 1995). β -catenin was first identified as a cytoplasmic ligand required for cadherin-mediated extracellular adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). Later, β -catenin was also found to be involved in Wnt family signal transduction (Gumbiner, 1995; Peifer, 1996). Further study showed that β -catenin's ability to regulate body axis determination was associated with its signal transduction function. β -catenin has also been found to form a complex with the tumor suppressor gene APC (Rubinfeld et al., 1993; Su et al., 1993; Polakis, 1995), a gene mutated in most cases of familial adenomatous polyposis. Both β -catenin and APC are in the Wnt

signal transduction pathway. APC seems to target β -catenin for degradation. In its absence, β -catenin accumulates, leading to colon cancer (Munemitsu et al., 1995). Additionally, β -catenin can bind to actin filaments at adherens junctions (Haftek et al., 1996), possibly through interactions with α -catenin, which binds to α -actinin (Knudsen et al., 1995; Rimm et al., 1995) and through direct interactions with fascin (Tao et al., 1996). Most recently, β -catenin was found to bind to the architectural transcription factor LEF-1 (Behrens et al., 1996; Molenaar et al., 1996). When associated with LEF-1, β -catenin translocates to the nucleus and might modulate the DNA-binding properties of LEF-1. β -catenin function was examined in transgenic knock-out mice, which produced ectodermal defects and no mesoderm formation at gastrulation (Haegel et al., 1995).

Although β -catenin has been cloned in several species, such as human, *Xenopus*, mouse and several invertebrates, it has not been isolated in chicken yet. Our lab is interested in the molecular basis of pattern formation. To explore further its possible role during development in the chicken model, we isolated chicken β -catenin from a cDNA library. The full-length chicken sequence was compared to the human, mouse and *Xenopus* sequence. Temporal and spatial expression of chicken β -catenin mRNA in chicken was examined in several stages of chicken embryos by both Northern blot and in-situ hybridization.

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Abbreviations: Wnt, wingless-int; FGF, fibroblast growth factor; AER, apical ectodermal ridge; APC, adenomatous polyposis coli; BMP, bone morphogenetic protein.

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1 GGCACGAGAG CAGCAGCAGC CGGAGCCCGG GGGGACGCTG CGCTCTCGCC TCCGTGCCAC AGTCTCTGAA AGGTAGCAGG AAAGAGTTCT GGGGACAGAG CAAGGAACAT GGCAACCCAA
N-terminal domain
A D L M E L D M A M E P D R K A A V S H W Q Q Q S Y L D S G I H S G A T T T A P
121 GCTGACTTGA TGAGTGTGA TATGGCCATG GAGCCAGACA GAAAAGCTCG AGTCAGTCAT TGGCAGCAGC AGTCATATCT GGACTCTGGT ATCCATTCGG GTGCCACGAC AACTGCTCCC
S L S G K G N P E E E D V D T T Q V L Y E W E Q G F S Q S F T Q E Q V A D I D G
241 TCTTTGAGTG GCAAAGGAAA TCCTGAAGAG GAAGATGTGG ACACAACGCA AGTCTCTGAT GAGTGGGAGC AAGGGTTTCT TCAGTCTCTT ACCCAGGAGC AAGTTGCTGA TATTGATGGC
Q Y A M T R A Q R V L S A M F P E T L D E G M Q I P S T Q F D A A H P T N V Q R
361 CAATATGCAA TGACTAGAGC TCAGAGAGTG CTTTCTGCTA TGTTCGCCGA AACACTGGAT GAAGGAATGC AAATCCCATC CACACAATTG GATGCTGGCC ATCCAACATA ATCGGACCGC
L A E P S Q M L K H A V V N L I N Y Q D D A E L A T R A I P E L T K L L N D E D
481 CTGCTGAGC CATCCGAGAT GCTAAAACAT GCTGTTGTTA ATTTGATAAA CTATCAGGAT GATGCTGAAC TTGCAACTCG TGCAATCCCA GAACTGACCA AACTGTTGAA TGATGAGGAC
Q V V V N K A A V M V H Q L S K K E A S R H A I M R S P Q M V S A I V R T M Q N
601 CAGGTGGTG TAAACAAGGC TGCGGTTATG GTTTCATCAGC TATCCAAAAA GGAAGCATCT CGCCATGCTA TTATGAGGTC TCCTCAAATG GTATCTGCAA TTGTGCGTAC CATGCAAAAT
T N D V E T A R C T A G T L H N L S H H R E G L L A I F K S G G I P A L V K M L
721 ACAAAAGCATG TGGAAACAGC CCGCTGTACT GCAGGCACAC TACACAATCT CTCACATCAC CGTGAAGGCT TGTGTGGCAAT CTTCAAATCA GGAGGCATCC CTGCGTTGGT TAAAATGCTT
G S P V D S V L F Y A I T T L H N L L L H Q E G A K M A V R L A G V L Q K M V A
841 GGGTCCCGG TGATTCTGT GTTGTCTATG GCCATTACTA CTCTTCACAA TCTCCTGTTA CATCAGGAGG GAGCCAAAAT GGCTGTCCGT CTGGCTGGAG GGCTGCAAAA AATGGTTGCC
L L N K T N V K F L A I T T D C L Q I L A Y G N Q E S K L I I L A S G G P Q A L
961 CTGCTCACA AGACAAATGT GAAATCTCTG GCCATCAGCA CACACTGTCT TCAGATTTTA GCCTATGGCA ATCAAGAAAG TAAGCTGATT ATTCTGGCAA GCGGTGGACC CCAAGCTCTA
V N I M R T Y T Y E K L L W T T S R V L K V L S V C S S N K P A I V E A G G M Q
1081 GTAAACATAA TGAGCCACTA CACTTATGAG AAATATTGTG GGACCACAAG TAGGGTGTCT AAGGTGTGTT CAGTCTGCTC CAGCAACAAA CCTGCTATTG TTGAGGCTGG TGGATGCAA
A L G L H L T D P S Q R L V Q N C L W T L R N L R H L S D A A T K Q E G M E G L L G T
1201 GCTTTAGGAC TCACCTTAC AGATCCAAAGC CAGCGTCTTG TCCAGAACTG TCTCTGGACC CTGAGAAATT TGTCAGATGC AGCAACCAAG CAGGAGGAA TGGAAAGGCT TCTAGGAACT
L V Q L L G S D D I N V V T C A A G I L S N L T C N N Y K N K M M V C Q V G G I
1321 CTGTTCAGC TTTTAGGATC AGATGACATT AATGTTGTA CTGCGCTGC CGGAATCCTT TCTAACCCTA CTTGCACABA TTACAAGAAC AAGATGATGG TCTGCCAGT TGGTGGCATC
E A L V R T V L R A G D R E D I T E P A I C A L R H L T S R H Q E A E M A Q N A
1441 GAGGCTCTG TGCCACAGT TCTTCGGGCT GGAGACAGG AGACATCAG AGAATCTGCT ATTTGTGCGC TCCGTCACCT CACCAGCAGA CATCAGBAAG CTGAAATGGC TCABAATGCA
V R L H Y G L P V V V K L L H P P S H W P L I K A A T V G L I R N L A L C P A N H
1561 GTACGCTCC ATTACGGACT CCGAGTGGTG GTTAAACTGT TGCACCCACC TCCACTGTC CCTTTGATCA AGGCTACTGT TGGGTGAT TGGGTGAT CGCAATCTCG CGCTGTGCC TCABAACAT
A P L R E Q G A I P R L V Q L L V R A H Q D T Q R R T S M G G T Q Q Q F V E G V
1681 GCGCCACTG GTGACBAGG TGCTATCCCA CGGCTAGTTC AGCTGCTGTT TAGAGCACAT CAAGATACC AGCGACGTCB TTCATGGGT GGAACGCAAC AGCAGTTTGT GGAGGGTGTG
R M E E I V E G C T G A L H I L A R D V H N R I V I R G L N T I P L F V Q L L Y
1801 GGCATGAGG AAATCGTGA GGGCTGACT GGAGCCCTGC ATATTCTGC ACCTGATGTT CACAATCGAA TTGTAATCAG GGGCTAAT ACCATCCAC TATTGTGCA GTTGTGTA
S P I E N I Q R V A A G V L C E L A Q D K E A A E A I E A E G G T A P L T E L L
1921 TCCCCATG AGAATATCCA GAGAGTAGCT GCGGGTGTAC TTTGTGACT TGCTCAGAC AAGGACAG CTGAAGCAAT TGAAGTGA GCGGAACTG CCCCTTAC AGAAGTCTT
H S R N E G V A T Y A A A V L F R M S E D K P Q D Y K K R L S V E L T S S L F R
2041 CATTCCAGG ATAGGGTGT TGCACATAT GCAGCTGAC TGCTGTTCAG AATGTCGGAG GACAAACAC AAGACTACA GAACCGACT TCAGTTGAAC TGACAAGCTC TCTTCCGG
T E P M A W N E T A D L G L D I G A Q G E P L G Y R P D D P S Y R S F H S G G Y
2161 ACTGAGCAA TGCTTGAA CGAGACAGCG GATCTGGAC TTGACATGG TGCCAGGGA GAACCTCTG GATACCGCC AGATGATCCT AGCTACGGT CTTCCACTC TGGCGGATC
G Q D A L G M D P M M E H E M G G H H P G A D Y P V D G L P D L G H A Q D L M D
2281 GGTGAGGAT CTTGGGTAT GGACCCATG ATGGAACATG AAATGGGTGG CCACCACCT GGTGCTGACT ACCCAGTTGA TGGTCTGCA GATCTGGCC ATGCCACGGA CCTTATGGAT
G L P P G D S N Q L A W F D T D L
2401 GGGCTGCCTC CAGGTGACAG TAATCAGTTG GCCTGGTTCG AACTGACCT GTAATATCAT CTTTAGCTGT ATCATCTGAA TGAACCTGCA TTGATTGGCC TGTAGAGTTG CTGAGAGGGC
2521 TCGAGGGGTG GGCTAGTATC TCAGAAAGTG CCTGACACAC TAACCAAGCT GAGTTTCTTA TGGGAACAAT TGAAGTAAAC TTTTGTCTT GGTCTTTTT GTGCGAGGAG TAATAATACA
2641 AATGATTTT GGGAGTATT CAAGAAACGA GGAATGCACA AGAATGAATT GCAAGATGGA ATTTATCAAA CCCTAGCCTT GCTTGTAAA AATTTATTAT TTTTAAA TCTCTGTAAT
2761 GGTACTGACC TTTGCTTCT TTGAAAGTAG CCTTCTTTT CGCAGTAATT GTTGTTAGG TTTTTTTTT AAGTCTCTCG TAGTATAAG TTATAGTAA TATGCTACG CCGTTCTAA
2881 TTTTAAAGA TTGAGTAAAG GTGTAGAACA CTAATCATA ATCGCTCTAA CTGATTCTG AATTAAGTGT AACATTGTGT AGCCTTTTT TATAAAAAA ACTAGACAAA TAGAATGGT
3001 CCAATTAGT TCCTTTTTAA TATGCTTAAA ATAAGCAGG GTATCTATTT CATGTTTTT ATCAAAACT TTATCTGGAT ATGCTGGGT AGGGCCAGT AAGAAGTGT TATTTGGAAC
3121 CTTGTATTG ACAGTTTACC AGTTGCCTT TATCCAAAG TTATTGTAGC CTGCTGTGAT ACAGATGCTT CATGAGAAA ATGCAGTTT AAAATGGTTC AAAATTAAAG TAAACTTTTA
3241 ATCAAAAAA AAAAAAAA AACTCGAGAG TACTTCTAGA G

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Fig. 1. Primary sequence of chicken β -catenin cDNA. The nucleotide and predicted amino acid sequences are shown. Nucleotide numbers are indicated to the left of the sequence. The amino terminus, armadillo repeats and carboxy-terminus are indicated above the sequence. The PCR product is underlined. The polyadenylation signal is bold. The nucleotide sequence reported here has been submitted to Genbank with the accession number U829621.

2. Results and discussion

2.1. Isolation of chicken β -catenin

Total RNA was isolated from the stage 31 chicken embryo dorsal skin by using TRIzol Reagent (Life

Technologies, Gaithersburg, MD, USA) and reverse-transcribed into cDNA using oligo-dT priming and AMV reverse transcriptase. A pair of degenerate primers were designed, based on a comparison of mouse and *Xenopus* β -catenin amino acid sequences. The forward primer was 5'-CAA/GATGC/TTNAAA/GCAC/TGC-3', and

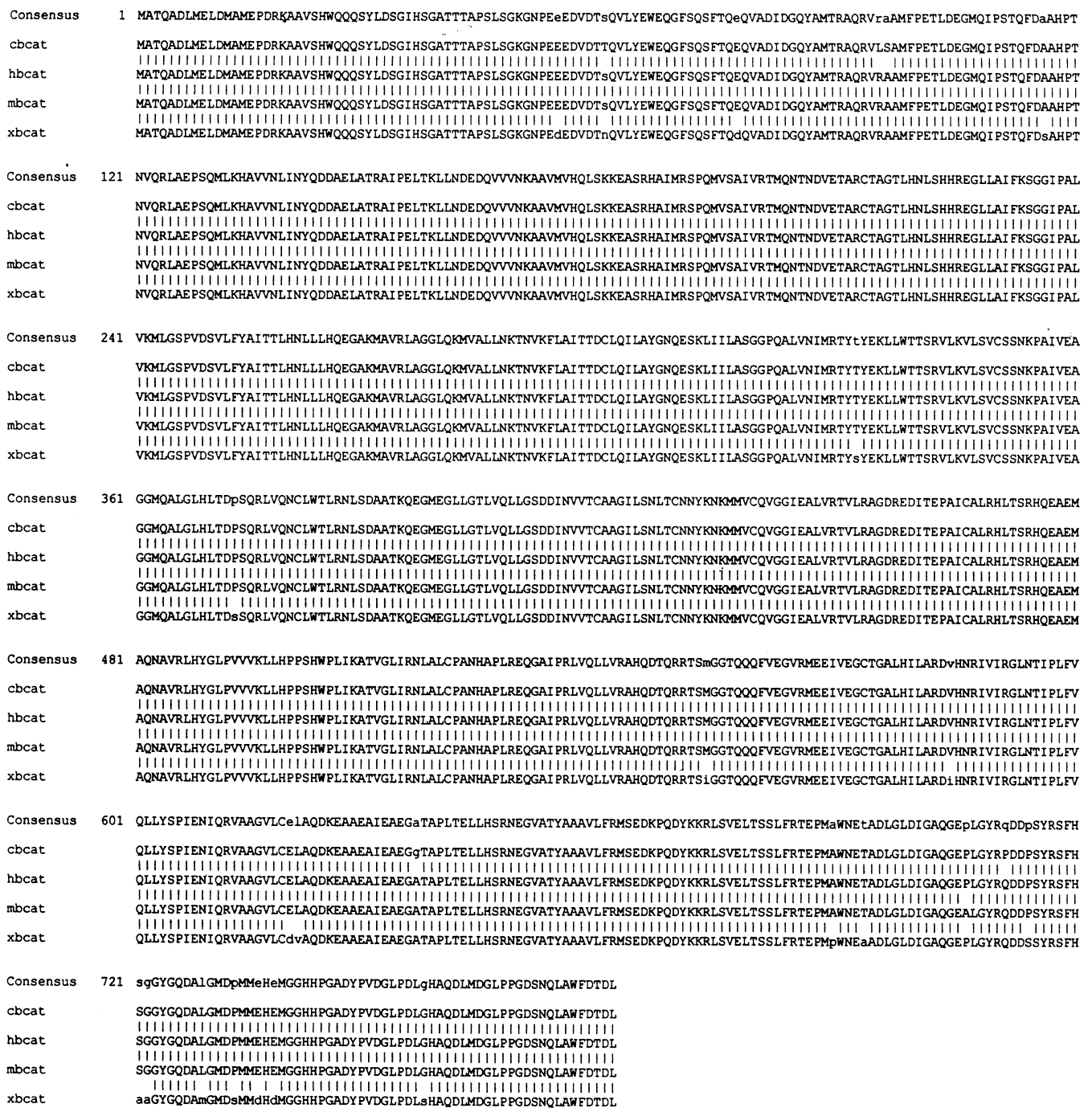


Fig. 2. Amino acid alignment of vertebrate β -catenin sequences. Predicted amino acid sequences of chicken, human, mouse and *Xenopus* β -catenin obtained from Genbank are compared. Capital letters in the consensus sequence (top row) indicate that the amino acid is conserved. cbcat, chicken β -catenin; hbcat, human β -catenin; mbcat, mouse β -catenin; xbcat, *Xenopus* β -catenin.

the reverse primer was 5'-A/GAAC/TATC/TAGG/AACNCCA/GAAC/TAG-3'. An 839-bp PCR fragment was subcloned into a TA cloning vector—PCR II—by using a TA cloning kit (Invitrogen, San Diego, CA, USA). Partial sequence data showed that it was a chicken homolog of β -catenin. This 839-bp fragment was then labeled with ^{32}P by random priming and used as a probe to screen a stage 29–34 chicken embryo library prepared

in the lambda Zap II vector kit (Stratagene, La Jolla, CA, USA). 1.5×10^5 plaques were screened. After three rounds of screening for plaque purification, we found eight positive plaques that were sequenced by the dideoxynucleotide sequencing method using the Sequenase v2.0 (US Biochemical, Cleveland, OH, USA). Results showed that one of the clones that was named clone 1–2b was a full length chicken β -catenin homolog. Five were incom-

plete chicken β -catenin cDNAs, and two were false positives. Several oligonucleotide sequencing primers corresponding to determined sequence were then generated to sequence the full length clone 1–2b. The full length sequence of clone 1–2b is shown in Fig. 1. It is 3.28 kb in length, containing an open reading frame of 781 amino acids with 108 bp of 5' UTR and 831 bp 3' UTR. In the 3' UTR, it contains the AATAAA poly A signal and some poly A tail.

The β -catenin sequence is highly conserved across species barriers. The chicken β -catenin sequence shares 99% amino acid sequence identity and 85% nucleotide sequence identity with the mouse and human sequences, and 97% amino acid identity with the *Xenopus* sequence (Fig. 2). Homology is particularly high in the armadillo repeat region (amino acids 130–693). Only one amino acid differs (aa 635) between the chicken, mouse and human sequences. The armadillo repeat region has been shown to bind to fascin, APC and E-cadherin (Hulsken et al., 1994; Tao et al., 1996) and is required for β -catenin function.

2.2. Expression of chicken β -catenin

To determine the size of β -catenin transcripts and to measure quantitatively the expression level of chicken β -catenin, we performed a Northern blot analysis using the β -catenin PCR fragment as a probe on poly A mRNA extracted from stage 31, stage 34, stage 38 and stage 41 chicken embryo (Fig. 3). Amounts of mRNA loaded in each gel lane were normalized by staining with Methylene Blue (data not shown). The 3.6-kb transcript was observed in every stage. In *Drosophila*, Armadillo transcripts are 3.2-kb (Riggleman et al., 1989) and in *Xenopus*, β -catenin transcripts are 3.5 kb (McCrea et al., 1991). The signal seems to be strongest at stages 34 and 38 and has dramatically decreased by stage 41.

β -catenin function has been studied extensively in *Xenopus* embryos where it contributes to dorsal–ventral axis formation. Its function in other species has not been studied. To begin to determine its role in the classical chicken limb model, we determined the distribution of β -catenin expression in stage 22, 24, 29 and 31 limbs by non-radioactive whole mount in-situ hybridization (Sasaki and Hogan, 1993). The TA cloning vector containing an 800-bp PCR product was linearized with XhoI and transcribed with SP6 RNA polymerase to make an antisense RNA probe. β -catenin is present in all of our tested stages of limb bud (Fig. 4). The transcript is localized in the apical ectodermal ridge (AER), and extends further along the anterior ridge than the posterior ridge of the limb bud. β -catenin expression is reduced and discontinuous along the AER at stage 31. To determine whether the β -catenin protein has a similar distribution, we stained fixed embryos with

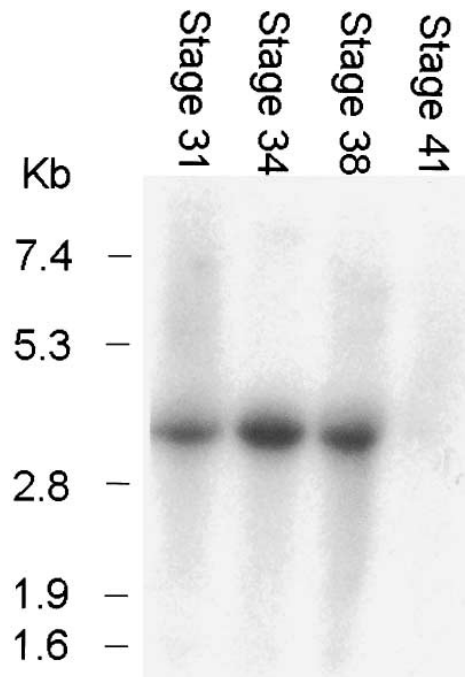


Fig. 3. Expression of the chicken β -catenin gene at different stages of embryogenesis. Poly(A)⁺mRNA was prepared from chicken embryos at stage 31, 34, 38 and 41. Sizes were compared to standard RNA markers (Boehringer-Mannheim, Mannheim, Germany). 4 μ g poly(A)⁺mRNA was electrophoresed on a 1% agarose gel containing 2 M formaldehyde. The RNA was transferred to a nylon membrane and hybridized to the PCR product labeled with ³²P by random priming. The symbols above each lane indicates the source of the mRNA.

an anti- β -catenin antibody (Funayama et al., 1995). The protein was distributed in a similar pattern as the mRNA (Fig. 4g). The AER is well known for its role as the signal center for controlling limb outgrowth through the underlying mesenchyme (Saunders, 1948; Summerbell, 1974). Recent studies also showed that the AER is very important for maintaining the activity of the polarizing region, a region of mesenchyme that is thought to provide the primary signal for patterning along the antero-posterior axis (Laufer et al., 1994; Niswander et al., 1994). Our results suggest that β -catenin might be involved in pattern formation along the proximodistal and anterior–posterior axes during limb development.

To date, fibroblast growth factor (FGF) family members FGF-2 (Savage et al., 1993; Dono and Zeller, 1994; Savage and Fallon, 1995), FGF-4 (Suzuki et al., 1992; Niswander and Martin, 1992) and FGF-8 (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Vogel et al., 1996), BMP-2, BMP-4 and BMP-7 (Lyons et al., 1990; Francis et al., 1994; Lyons et al., 1995), Wnt 5a and Wnt 12 have been found to be expressed in the AER of mouse and chicken during limb development (Dealy et al., 1993; Christiansen et al., 1995). Since β -catenin is involved in

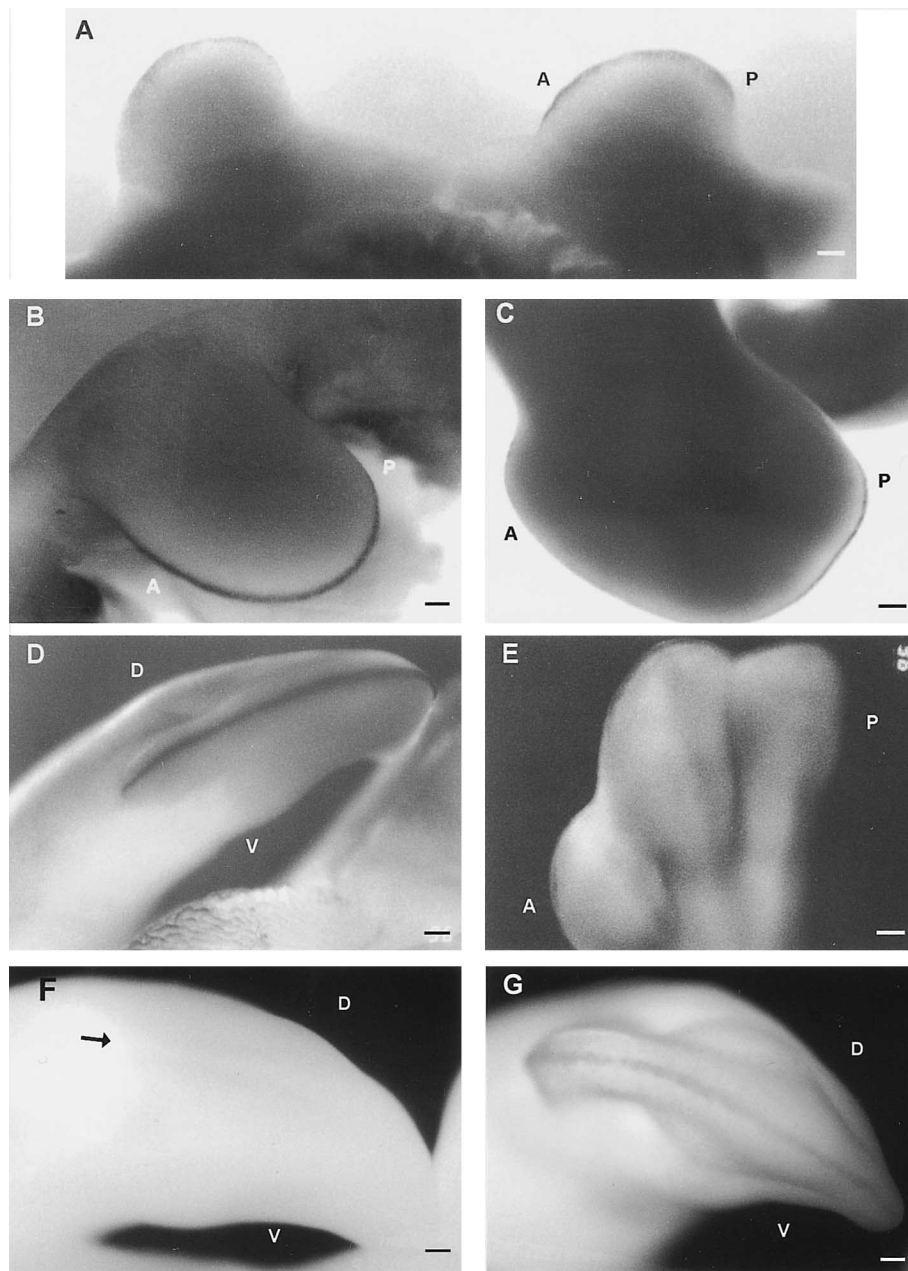


Fig. 4. Spatial and temporal expression of the β -catenin transcript in chick limb bud development. Note the absence of β -catenin in the proximal limb bud and trunk ectoderm. Abbreviations: A, anterior; P, posterior; D, dorsal; V, ventral. Size bars = 250 μ m. (A) Dorsal view of stage 22 chicken limb bud: β -catenin is expressed in the AER and extends further along the anterior AER than the posterior AER. (B) Dorsal view of stage 24 chicken wing bud: β -catenin is localized in the AER. (C) Dorsal view of stage 29 chicken leg bud: β -catenin transcripts are expressed in AER extending further along the anterior end than the posterior end. (D) Anterior view of stage 29 chicken wing bud: β -catenin is specifically expressed in the AER. (E) Ventral view of stage 31 chicken leg bud: β -catenin is expressed discontinuously at low levels in the AER, covering only the digit regions. (F) Control whole-mount immunostaining of stage 28 chicken wing (anterior view) with non-immune serum. Staining is detected with alkaline phosphatase conjugated secondary antibodies. The AER is indicated by the arrow. (G) Whole-mount immunostaining of stage 28 chicken wing (anterior view) with anti- β -catenin antibodies (Funayama et al., 1995). Staining is detected with alkaline phosphatase conjugated secondary antibodies. Note the strong staining in the AER.

Wnt family signal transduction, the partial colocalization of β -catenin with Wnt family members suggests that β -catenin might be involved in transmitting the Wnt 5a and/or Wnt 12 signal in the AER. L-CAM protein (likely chicken E-cadherin) was shown to be in the whole limb bud ectoderm, including the AER

(Crossin et al., 1985). The enrichment of β -catenin in the AER may contribute to its unique function. β -catenin is also strongly expressed in other sites of epithelial–mesenchymal interactions; its expression in the limb was presented as an example of its expression pattern.

3. Conclusion

Here, we report the cloning and characterization of chicken β -catenin. Our results reveal that β -catenin is highly conserved across species barriers, especially in the armadillo repeats, perhaps due to its extensive interactions with other proteins, including cadherin, APC, the Wnt pathway, fascin and LEF-1. Since β -catenin is expressed in the AER of the limb bud during development, we expect that it is important in forming the proximal–distal and possibly the anterior–posterior axes during limb bud morphogenesis.

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