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Identification and Characterization of a Soluble Cadherin-7 Isoform Produced by Alternative Splicing*

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We identified an alternative mRNA encoding a novel cadherin-7 isoform by reverse transcriptase-PCR of RNA from day 12 chicken embryos. The alternative mRNA contains 49 bases of insertion in the premembrane region, leading to the substitution of 14 amino acids and the introduction of a premature stop codon. Identification of a 49-bp insertion sequence in the genomic DNA corresponding to the intron of the cadherin-7 gene suggests that alternative splicing is the cause of the alternative mRNA. Transient expression of the variant form in COS-7 or 293 cells produced a soluble protein. Aggregation assays and immunoprecipitation showed that the variant protein interacts with full-length cadherin-7 *in vitro* and *in vivo* and inhibits full-length cadherin-7-mediated cell adhesion. Immunohistochemistry revealed that the variant form was strongly expressed in dermomyotomes rather than in migrating neural crest cells, in contrast to the full-length cadherin-7, suggesting differential regulation of splicing and possible roles of variant cadherin-7 in the development of dermomyotomes and other tissues.

Cell adhesion molecules are usually transmembrane glycoproteins that mediate binding interactions at the extracellular surface and determine the specificity of cell-cell recognition. The cadherin, integrin, immunoglobulin, and selectin families are major cell adhesion molecules (1). The molecules of the cadherin family mediate calcium-dependent cell-to-cell adhesion, and they play roles in cell recognition and cell sorting during development (2, 3). They can be divided into six major subfamilies: 1) classic (type I) cadherins, 2) atypical (type II) cadherins, 3) desmocollins, 4) desmogleins, 5) protocadherins, and 6) Flamingo cadherins, and several cadherins clearly occupy isolated positions in the cadherin superfamily (4).

The type I (classic) and type II (atypical) cadherins have a characteristic structure, with NH_2-terminal domain, four CRs1 (CR1–CR4),[1] a pretransmembrane domain (CR5), a transmembrane domain, and a highly conserved cytoplasmic domain (2, 5), except for T-cadherin (6, 7). CR1 provides the binding specificity of cadherins (8, 9). The type I cadherins have an HAV motif in the CR1 domain, and this motif is important for calcium-dependent adhesion (10). The type II cadherins, on the other hand, have no HAV site. This difference may be responsible for the weaker intercellular adhesion shown by type II compared with type I cadherin (11). The highly conserved cytoplasmic domain, which associates with α- and β-catenin, plakoglobin, and p120, is responsible for the stabilization of cell-to-cell adhesion (12–16).

Cadherin-7, a type II cadherin, is expressed in some populations of migrating neural crest cells, proximal dorsal and ventral roots, and dorsal root ganglia in chicken embryos. It is also expressed in non-neural crest cells, such as motor neurons, and cells in the floor plate and in myotomes (5). Overexpression of cadherin-7 blocks neural crest segregation from neuroepithelial cells (17). The regulated expression of cadherins, including cadherin-7, in the neuroepithelium is essential to maintain its integrity and to send out neural crest cells in the correct direction (17). Cadherin-7 shows a partial affinity to cadherin-6B, but the heterophilic interaction is less stable than the homophilic (5). Cadherin-7 is involved in intercellular signaling between homotypic cells and in the sorting of heterotypic cells, but the precise function of cadherin-7 has not yet been determined (17, 18).

Splicing variants of type II cadherin have been reported for cadherin-11 (19) and PB-cadherin (20). These variant forms are truncated transmembrane protein with a cytoplasmic domain that differs from full-length forms. In this report, we identified a soluble splicing variant form of chicken cadherin-7. Competitive PCR analysis and immunostaining showed differential expression patterns between the variant form and full-length cadherin-7. The variant protein interacted with and inhibited cell-to-cell adhesion mediated by full-length cadherin-7 *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Animals—Fertilized eggs of White Leghorn chickens were purchased from commercial sources. They were incubated at 38 °C, and staging of the embryos was performed according to Hamburger and Hamilton (21). Rabbits were purchased from commercial sources for the production of polyclonal antibodies (Abs).

Isolation of Variant Form of Chicken Cadherin-7 cDNA—Total RNA was prepared from embryonic day (E) 12 chicken brains using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan), and cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). PCR primers were designed based on the chicken full-length cadherin-7 cDNA sequence (GenBank™ accession number D42150) as follows: U1847 (see Fig. 1, *B* and *C*), 5′-TGGCTATGGAGAGTCAGAATCC-3′; L2158 (see Fig. 1, *B* and *C*), 5′-CAGATTGTTCCTGTCTTCGG-3′. The cDNA was amplified with these primers by PCR. The

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY115555.

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[1] The abbreviations used are: CR, cadherin repeat; Ab, antibody; HRP, horseradish peroxidase; PB-cadherin, pituitary gland and brain-cadherin; P-cadherin, placental-cadherin; T-cadherin, truncated-cadherin; E-cadherin, epithelial-cadherin; E, embryonic day; PBS, phosphate-buffered saline; HCMF, hepes-buffered calcium and magnesium-free Hank's solution.

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PCR products were separated using 4.0% agarose gel electrophoresis. The amplified fragment (360 bp) was cloned into the pGEM-T Easy Vector (Promega) and sequenced on an ABI 310 sequencer (PerkinElmer Life Sciences and Applied Biosystems).

Cloning of the Partial Chicken Cadherin-7 Gene—Chick genomic DNA was extracted from whole E6 embryos according to the standard protocol (22). Modified nested PCR of the genomic DNA was performed as follows. The chicken genomic DNA was first amplified using primers U1593 (see Fig. 3*A*), 5′-AGAAGATGTTGATGAGCCACC-3′, and L2158, and the PCR product was further amplified with primers U1861 (see Fig. 2), 5′-CAGAATCCAGCACAGATTGG-3′, and L2158, using Ex *Taq* polymerase (Takara, Tokyo, Japan). The PCR cycle conditions were 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 10 min, followed by 72 °C for 10 min. The PCR product of about 6 kbp was cloned into the pGEM-T Easy Vector and sequenced.

Competitive PCR for Quantitative Analysis—To develop a competitive PCR assay for variant and full-length chicken cadherin-7, a competitor DNA was prepared. The variant form cDNA was amplified by PCR using two primers, U1593 and L2158. The amplified 634-bp fragment was cloned into the pGEM-T Easy Vector. The DNA was cut with *Nco*I (Takara) and re-ligated to delete a 249-bp *Nco*I fragment (Fig. 3*A*). The copy number of competitor molecules was determined, and then 10-fold serially diluted competitor DNAs were added to PCR reactions. Total RNAs were prepared from each stage of the chicken embryos using Isogen, and first-strand cDNAs were synthesized from 1 μg of total RNAs in 20 μl of reaction mixture using SuperScript II reverse transcriptase. The combinations of PCR primers are shown in Fig. 3*A*. The nucleotide sequence of the primer L variant was 5′-TTGACCGCTTCTGAGAGGTAGG-3′, and the sequences of the other primers are described above. Competitive PCR was performed in 20 μl of reaction mixture containing 0.2 mM each of the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1 μl of each 10-fold serially diluted competitor, and 1 unit of rTaq polymerase (Takara) with *Taq* start antibody (Clontech). The PCR cycle conditions were 94 °C for 2 min, 30 cycles of 94 °C for 40 s, 58 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and observed under UV illumination.

Expression of Chicken Full-length Cadherin-7 in L Cell Lines—To obtain a cDNA clone coding for full-length cadherin-7, a cDNA phage library from E9 chicken brain in the vector lambda ZapII (Stratagene) was screened with a radiolabeled probe for the cadherin-7 fragment (350 bp). The phage clone containing the cDNA for full-length cadherin-7 was subcloned into the plasmid vector, Bluescript II SK+ (pBS-cad7) (Toyobo, Tokyo, Japan). After digestion with *Eco*RI, a 3-kb fragment containing the complete coding sequence was recloned into a eukaryotic expression vector, pCAGGS (23), to obtain pCA-cad7. L cells were transfected with pCA-cad7 using LipofectAMINE Plus reagent (Invitrogen). Transfectants were screened by immunoblotting and immunostaining using CCD7–1, and a stable cell line expressing cadherin-7 (L-cad7) was thus obtained.

Expression of the Variant Form of Chicken Cadherin-7 in COS-7 or 293 Cells—The expression vector for cadherin-7 variant was constructed as follows. cDNA from E12 chicken brain was amplified with two primers, U427 (see Fig. 1*C*) designed in the 5′-untranslated region, 5′-AGAGACAACTGGAATTGTGCC-3′, and L2158 (see Fig. 1, *B* and *C*). Amplified fragments contained both the full-length and variant cDNAs and were cloned in pGEM-T Easy Vector. A single-clone plasmid containing variant cDNA was selected by PCR using two primers, U1847 (see Fig. 1, *B* and *C*) and L variant (see Fig. 3*A*), and verified by sequencing. The selected plasmid, pPCR-v, was amplified with two primers, U427, and Lxba I, 5′-GCTCTAGAATACAGGTTGACCGCTTC-3′. The primer of Lxba I was designed to remove the stop codon and introduce an *Xba*I site at the 3′-terminal end. The amplified U427-Lxba I fragment was cloned in pGEM-T Easy Vector and recloned into pEF-1/Myc-His A (Invitrogen) to generate the expression construct pEF-v. COS-7 or 293 cells were transfected with pEF-v (COS variant cells or 293 variant cells), and 293 cells were transfected with pEF-1/Myc-His A (293 mock cells) as control by LipofectAMINE Plus reagent. Following incubation with serum-free RPMI 1640 medium (Sigma) for 2 days, culture media were concentrated using Centricon YM30 tubes (Amicon) and replaced with 50 mM Tris-HCl (pH 7.5). Media were finally concentrated up to about 30-fold. We confirmed that the expression level of variant protein was almost equal with the COS variant and 293 variant cells (data not shown).

Aggregation Assays—Monolayer cultures of L-cad7 were treated with 0.01% Trypsin (Sigma) and 10 mM $CaCl_2$ in Ca^{2+}- and Mg^{2+}-free saline buffered with 10 mM HEPES (HCMF, pH 7.4) for 30 min at 37 °C. The treated cells were washed three times in HCMF with 1 mM EDTA and dissociated into single cells (24). The dispersed cells were suspended in HCMF with 1 mM $CaCl_2$ or 1 mM EDTA at a density of 2 × 105 cells/ml. 24-well plates (Iwaki, Tokyo, Japan) were coated with 1% bovine serum albumin (Sigma) for 1 h at room temperature and washed twice with HCMF. The cell suspension (0.5 ml) was dispensed into each well with 30-fold-concentrated culture medium from COS variant cells or COS-7 cells. The plates were incubated at 37 °C on a gyratory shaker rotating at 40 rpm.

Antibodies—Antibodies specific to the variant form of cadherin-7, anti-variant Ab, were generated in rabbits by immunizing with the COOH-terminal peptide of the variant form, NH_2-GDVSPYLSEAVNLY-COOH (Fig. 1*B*), conjugated with Keyhole Limpet hemocyanin as described before (25). The antiserum was affinity-purified with an epoxy-activated Sepharose 6B column (Amersham Biosciences) that was modified with the variant peptide. The other antibodies used in the experiment were as follows: mouse IgG monoclonal antibody against cadherin-7; CCD7–1 (a generous gift from Drs. S. Nakagawa and M. Takeichi of Kyoto University); HRP-conjugated anti-mouse IgG (Wako, Osaka, Japan); biotinylated anti-mouse IgG (Wako); HRP-conjugated anti-rabbit IgG (Wako); and Texas Red-conjugated streptavidin (Caltag).

Immunoblotting Analysis—The concentrated culture media of L-cad7, 293 variant and 293 mock cells were mixed with 2× sample buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2% SDS, 20% sucrose, 1.2% mercaptoethanol, 0.01% BPB). These cells were lysed with 1× sample buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 10% sucrose, 0.6% mercaptoethanol, 0.005% BPB). The samples were boiled for 5 min and run in 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with appropriate antibodies, and the signals were detected using the ECL or ECL Plus systems (Amersham Biosciences).

Immunoprecipitation—The L-cad7 cells were dissociated into single cells as described in the methodology for the aggregation assay and re-aggregated in culture medium of COS variant cells in the presence of $CaCl_2$ for 15 min. The re-aggregated cells were lysed in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1% trypsin inhibitor) with 1% Triton X-100.

E5 chicken embryos were homogenized in lysis buffer without Triton X-100. The tissue lysate was centrifuged at 70 × *g* for 5 min at 4 °C. The supernatant (whole homogenate) was centrifuged at 65,000 × *g* for 1 h at 4 °C, and fractionated into the supernatant (soluble fraction) and precipitate. The precipitate was suspended in lysis buffer with 1% Triton X-100 and centrifuged at 14,000 × *g* for 5 min at 4 °C. The supernatant (membranous fraction) was then collected.

For immunoprecipitation, the cell lysate and whole homogenate with 1% Triton X-100 was incubated with a 1/10 volume of purified anti-variant Ab at 4 °C for 5 h, then protein G beads (Amersham Biosciences) were added, and the mixture was incubated at 4 °C for 1 h. The beads were washed three times with lysis buffer with 1% Triton X-100 and dissolved in sample buffer. Bound proteins were eluted from the beads by boiling for 5 min.

Immunostaining—Cultured cells were fixed and permeabilized in 100% methanol with 3% H_2O_2 for 1 h at room temperature. They were incubated with CCD7–1 antibody or anti-variant Ab, followed by incubation with HRP-conjugated second antibody. For the detection of HRP, Tyramid reagent (PerkinElmer Life Sciences) was used. For double-label immunostaining, they were incubated with CCD7–1 antibody, followed by biotinylated anti-mouse IgG and Streptavidin-Texas Red, and then with anti-variant Ab, followed by anti-rabbit IgG-HRP and Tyramid reagent.

E5 chicken embryos were fixed in 4% paraformaldehyde at 4 °C for 2 h and washed in PBS at 4 °C overnight. They were permeabilized in a graded series of methanol in PBS (25, 50, 75, and 100% at 4 °C for 30 min each), placed in 6% H_2O_2 in methanol at room temperature for 2 h, and re-hydrated in PBS. Nonspecific binding of antibodies was blocked by immersion in 10% skim milk with 0.01% Triton X-100 in PBS (PBSMT) at room temperature for 2 h. Embryos were incubated with CCD7–1 antibody or anti-variant antibody at 4 °C overnight. The embryos were washed in 0.01% Triton X-100 in PBS (PBST) for 6 h at 4 °C, immersed in PBSMT for blocking of nonspecific binding of antibody, and then incubated with an appropriate HRP-conjugated second antibody at 4 °C overnight. The embryos were washed in PBST for 6 h at 4 °C. For the detection of HRP activity, they were immersed in PBS containing 166 μg/ml 3,3′-diaminobenzidine at room temperature for 30 min, and H_2O_2 was added to a final concentration of 0.003%. Embryos were dehydrated in a graded series of ethanol, followed by 100% chlo-

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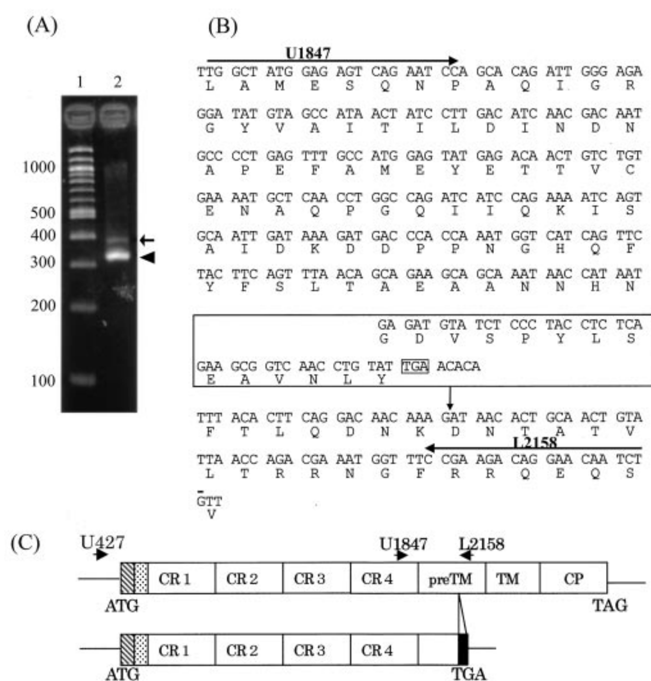

FIG. 1. **Identification of the variant form of chicken cadherin-7.** *A*, RT-PCR analysis on total RNA from day 12 embryonic chicken brains. *Lane 1*, 100-bp ladder maker; *lane 2*, the RT-PCR products. An *arrow* and an *arrowhead* indicate the amplified DNA fragments of variant and full-length chicken cadherin-7, respectively. In the reaction, primers U1847 and L2158 were used for the amplification of cDNA encoding a partial CR4 domain and pretransmembrane fragment of cadherin-7. *B*, partial nucleotide sequences and predicted amino acid sequences of chicken full-length cadherin-7 and the variant form. The *large* and *small boxes* indicate a 49-bp insertion and termination codon, respectively. The *horizontal arrows* show the binding sites for the two primers, U1847 and L2158, used in the PCR reaction. *C*, schematic representation of chicken full-length cadherin-7 (*top*) and the variant form (*bottom*). The *boxes* and *horizontal lines* indicate the coding and untranslated regions, respectively. Each *box* is as follows: *hatched box*, precursor region; *dotted box*, NH_2-terminal domain; *CR1–4*, cadherin repeats 1–4; *preTM*, pretransmembrane domain; *TM*, transmembrane domain; *CP*, cytoplasmic domain; *closed box*, the COOH-terminal segment of the variant form of cadherin-7. *ATG*, *TAG*, *TGA*, and *arrows* indicate first methionine of translation, stop codons, and the binding sites of primers used for PCR, respectively.

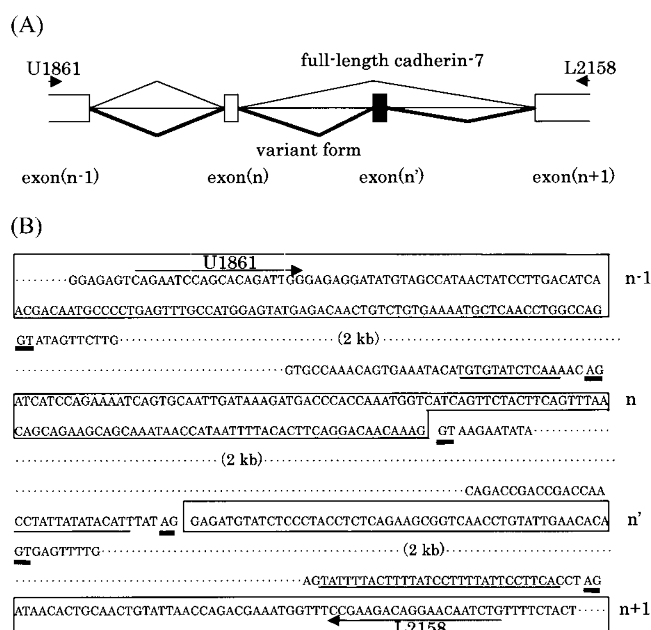

FIG. 2. **Partial structure and nucleotide sequence of the chicken cadherin-7 gene.** *A*, schematic representation of the cadherin-7 genomic organization leading to two alternative splicings: the variant form and full-length cadherin-7. About 6 kbp of genomic DNA fragment was cloned from chicken embryo by the PCR method. The *arrows* indicate the primers used, U1861 and L2158. *B*, the partial nucleotide sequence of genomic DNA of chicken cadherin-7. The nucleotide sequences representing the exons are *boxed*. The mRNA of variant form only includes the additional exon (*n′*) sequence. The *horizontal bars* and *underlines* indicate the GT/AG sequences in the splicing sites, and the polypyrimidine tract adjacent to AG dinucleotides, respectively.

roform, 50% paraffin in chloroform, and then embedded in paraffin. Serial 10-μm sections were cut on a microtome, de-waxed, and mounted on glass slides using Permount (Fisher).

RESULTS

Identification of a Variant Form of Chicken Cadherin-7—Two DNA fragments were detected by PCR reaction on cDNA from E12 chicken brain using primers that amplified the cDNA encoding for partial CR4 and the pretransmembrane domain of cadherin-7 (Fig. 1*A*). Nucleotide sequence data showed that the longer fragment had the partial cDNA sequence of full-length cadherin-7 and an insert sequence of 49 bp, which caused frameshift and a stop codon within the insert, and the shorter fragment corresponded to the partial cDNA sequence of full-length cadherin-7 (Fig. 1*B*). Because the stop codon existed before the transmembrane domain, the variant transcript coded for the soluble form of cadherin-7, possessing only the extracellular domains of full-length cadherins (Fig. 1*C*).

To examine the origin of the 49-bp insert and exon-intron junction in the chicken genome, a genomic DNA fragment of ~6 kbp, coding for the CR4 and pretransmembrane region, was cloned by PCR. The DNA clone was sequenced, and four exons were detected (Fig. 2*B*). Consensus GT and AG sequences, including the adjacent polypyrimidine tract were found in the 49-bp insertion (26). All data suggested that the variant form of cadherin-7 had been generated by alternative splicing (Fig. 2*A*).

Expression Level of mRNA of the Variant Form in Chicken Embryos—We carried out competitive PCR to compare the transcription levels of the variant form and full-length cadherin-7 at different developmental stages (Fig. 3). The expression level of the variant form was very low in whole embryos at stage 10, compared with that of the full-length cadherin-7. Because neural crest migration, somitogenesis, and myogenesis in the rostral region precede those in the caudal region, we examined the expression level of the variant form and full-length cadherin-7 in rostral and caudal halves of chick embryos at stage 17 separately. A significant expression of variant mRNA was detected in the rostral half of the trunk, although expression in the caudal half was still low. The amounts of variant transcripts in the rostral half were in the range from one-tenth to one-fifth of that of full-length cadherin-7. After this stage, the expression level of variant form in the trunk at wing level was almost constant until E9, maintaining the same ratio to that of full-length cadherin-7 (Fig. 3*C*).

Expression of the Variant and Full-length Forms of Cadherin-7 in Vitro—To obtain information on the variant protein, we generated anti-variant Abs that specifically recognized the variant form of cadherin-7 by immunizing rabbits with the short COOH-terminal peptide. We introduced the cDNA of the variant form into COS-7 or 293 cells (COS or 293 variants) transiently and the cDNA of full-length cadherin-7 into L cells (L-cad7) stably. The expressions of full-length cadherin-7 and the variant form were confirmed by immunostaining with CCD7–1 and anti-variant Ab (Fig. 4*A*). It is noteworthy that full-length cadherin-7 signal was detected at the cell boundary, indicating that it was functioning in cell-to-cell interaction,

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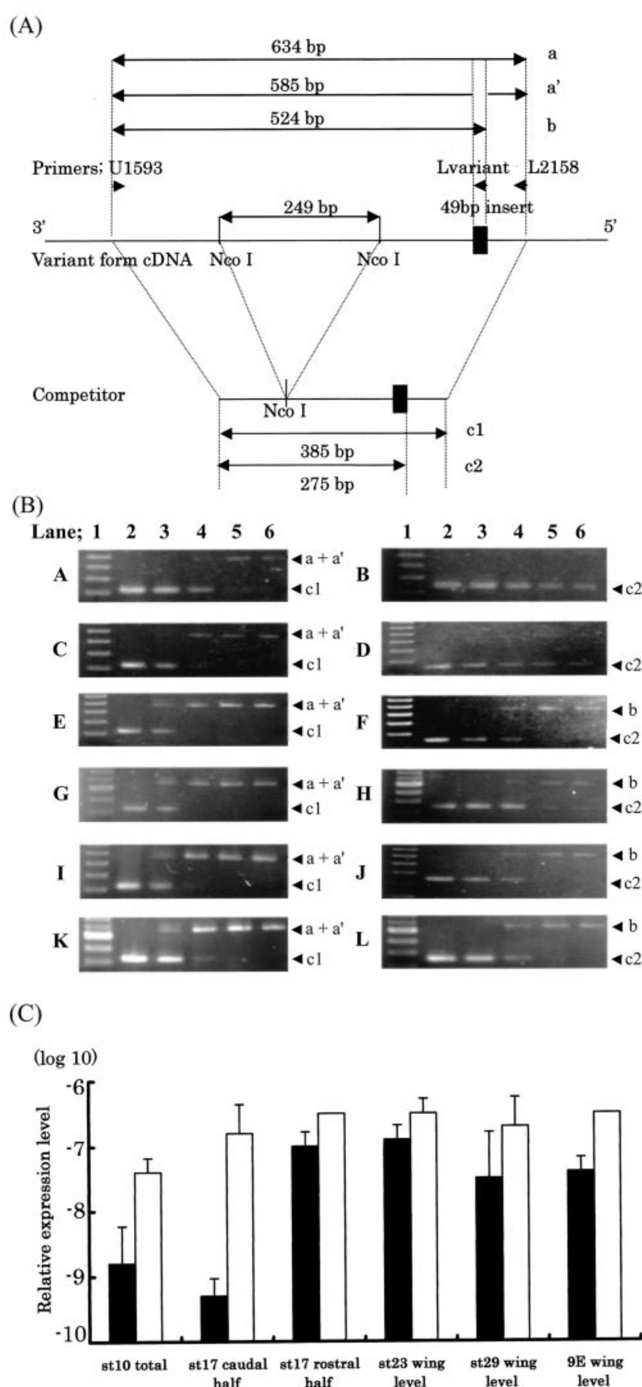

FIG. 3. **Competitive PCR analysis of the variant form and full-length cadherin-7.** *A*, schematic representation of amplified fragments and the competitor. The *letters* indicate the following: *a* and *a′*, amplified fragments of the variant and full-length form using U1593 and L2158; *b*, an amplified fragment of the variant form using the primers U1593 and L variant; *c1* and *c2*, amplified fragments of the competitor using the primers U1593 and L2158, and U1593 and L variant, respectively. *B*, agarose gel analysis of competitive PCR products using RNAs from chicken embryonic tissues. Each gel shows a representative experiment from five similar results. The sources of RNAs are as follows: *A* and *B*, total embryo at stage 10; *C* and *D*, caudal half trunk at stage 17; *E* and *F*, rostral half trunk at stage 17; *G* and *H*, trunk at wing level at stage 23; *I* and *J*, trunk at wing level at stage 29; *K* and *L*, trunk at wing level at 9E. The combination of primers U1593 and L2158 was used in *A*, *C*, *E*, *G*, *I*, and *K*, whereas that of U1593 and L variant were used in *B*, *D*, *F*, *H*, *J*, and *L*. The lanes contain the products amplified from a constant amount of cDNA mixture, and the competitor DNA was prepared in successive 10-fold dilutions. *a*, *a′*, *b*, *c1*, and *c2* indicate the amplified DNA fragments. Note that the fragments *a* and *a′* showed almost the same mobility on the 2% agarose gel. *C*, quantification of the results of competitive PCR. The values represent the means ±S.D. values of five separate experiments. The *black* and *white bars* indicate the relative expression level of the variant and full-length forms, respectively. The results were demonstrated as -fold relativity. glyceraldehyde-3-phosphate dehydrogenase levels of each sample were not different (data not shown).

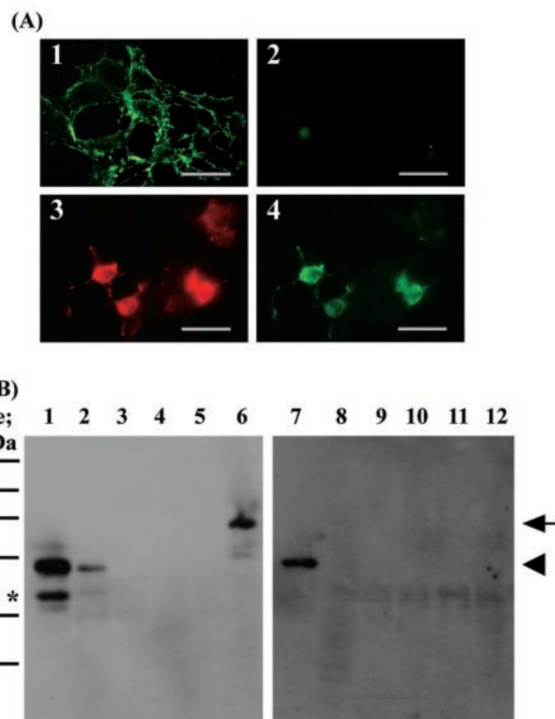

FIG. 4. **Immunostaining and immunoblotting of the variant and full-length cadherin-7-expressing cells.** *A*, immunofluorescent staining of L-cad7 (*1* and *2*) and COS variant (*3* and *4*). Cells were stained with CCD7–1 (*1* and *3*) and anti-variant Ab (*2* and *4*). The signals in *2* were nonspecific, and in *3* and *4* the same cells were double-stained. *Bars*, 50 μm. *B*, immunoblotting analysis using CCD7–1 (*lanes 1–6*) and anti-variant Ab (*lanes 7–12*). The concentrated conditioning medium of 293 variant (*lanes 1* and *7*), 293 mock (*lanes 3* and *9*), L-cad (*lanes 5* and *11*), and whole cell lysate of the 293 variant (*lanes 2* and *8*), 293 mock (*lanes 4* and *10*), L-cad7 (*lanes 6* and *12*) were applied. The *arrow*, *arrowhead*, and *asterisk* indicate full-length cadherin-7 (105 kDa), the variant protein with myc-His tag (73 kDa), and degradation products of the variant protein, respectively. The protein marker represents a pre-stained protein marker broad range (New England BioLabs).

whereas the signal for the variant form was found inside the cell body.

Immunoblotting, using CCD7–1 and the anti-variant Ab, detected the 73-kDa band of the variant form with an Myc-His tag in the concentrated culture medium of 293 variant cells (Fig. 4*B*, *lanes 1* and *7*). In the cell lysate of 293 variant, CCD7–1 detected this band weakly (Fig. 4*B*, *lane 2*), but anti-variant Ab did not detect it (Fig. 4*B*, *lane 8*), because the sensitivity of anti-variant Ab was lower than that of CCD7–1. It was thought that the lower bands of *lanes 1* and *2* (Fig. 4*B*) were degradation products. CCD7–1 detected the 105-kDa band of full-length cadherin-7 in the lysate of L-cad7 cells (Fig. 4*B*, *lane 6*), but anti-variant Ab detected no band (Fig. 4*B*, *lane 12*). Full-length cadherin-7 expressed by L-cad7 was highly degradable during lysis procedures as previously reported (17). The lower band of *lane 6* (Fig. 4*B*) reflects these degradation products. Because full-length cadherin-7 was a membranous protein, CCD7–1 did not detect it in the concentrated culture medium of L-cad7 (Fig. 4*B*, *lane 5*). Anti-variant Ab detected no band in the concentrated culture medium of L-cad7 (Fig. 4*B*,

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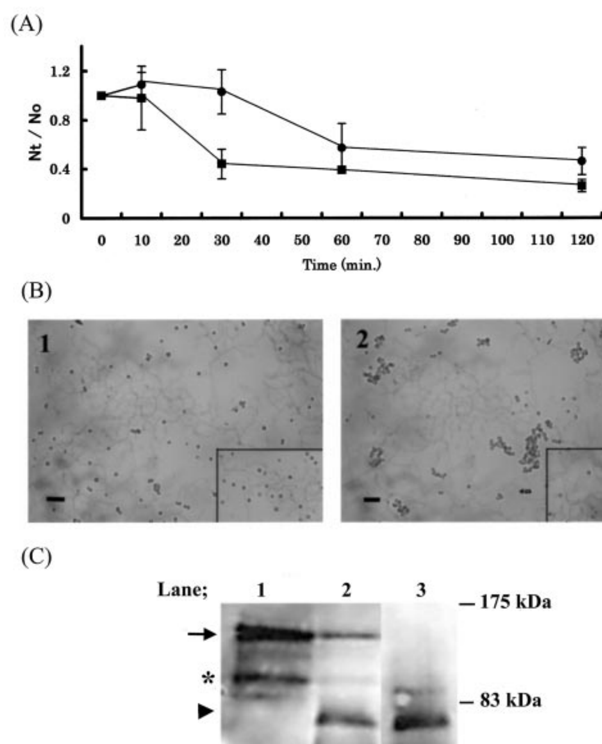

FIG. 5. **Effects of the variant protein on cell aggregation mediated by full-length cadherin-7.** *A*, time course of cell aggregation mediated by full-length cadherin-7 with or without the variant protein. L-cad7 cells were dissociated by trypsin treatment and were allowed to aggregate in conditioning medium from COS variant cells that contained the variant protein (*closed circles*) or COS-7 cells (*closed squares*) in the presence of 1 mM $CaCl_2$. The aggregation was represented by the index Nt/No, where No is the initial cell number before aggregation and Nt is the total particle number at incubation time t (minutes). Values represent the means ±S.D. of six separate experiments. *B*, appearance of L-cad7 cells in aggregation assays with or without the variant protein. The L-cad7 cells were aggregated for 30 min in conditioning medium from COS variant cells (*panel 1*) or COS-7 cells (*panel 2*) with 1 mM $CaCl_2$. *Insets* show mock experiments in the absence of $CaCl_2$. *Bars*, 50 μm. *C*, co-immunoprecipitation of the variant protein and full-length cadherin-7. After an aggregation assay was performed with the variant protein and 1 mM $CaCl_2$ for 15 min, the cell lysate was immunoprecipitated with anti-variant Ab. The precipitates were loaded onto the SDS-polyacrylamide gel for immunoblotting analysis (*lanes 2* and *3*). The filter was incubated with CCD7–1 (*lane 2*) or anti-variant Ab (*lane 3*). For comparison, the lysate of L-cad7 cells was loaded, blotted, and incubated with CCD7–1 (*lane 1*). The signals were detected using an ECL system. The *arrow*, *arrowhead*, and *asterisk* indicate full-length cadherin-7, the variant protein with the myc-His tag, and degradation products of full-length cadherin-7, respectively.

lane 11). CCD7–1 and anti-variant Ab detected no band in the lysate and concentrated medium of 293 mock cells.

Effect of the Variant Form on Cell Adhesion Mediated by Full-length Cadherin-7 in Vitro—We introduced a cell aggregation assay mediated by full-length cadherin-7 using L-cad7 cells to examine a possible function for the variant protein (5). The aggregation of L-cad7 cells was significantly slower in the presence of the conditioned medium from COS variant cells than in that of COS-7 cells within 30 min. Even after 120 min, the difference was still apparent. These aggregations were calcium-dependent (Fig. 5, *A* and *B*). These data suggest that the variant form inhibits cell aggregation mediated by full-length cadherin-7.

We performed immunoprecipitation to confirm interaction of the variant protein with full-length cadherin-7. We collected and lysed the cells after 15-min aggregation, and immunoprecipitation was performed using anti-variant Ab. The full-length cadherin-7 co-immunoprecipitated with the variant protein as shown in Fig. 5*C*. These results indicate that the variant protein interacts with full-length cadherin-7.

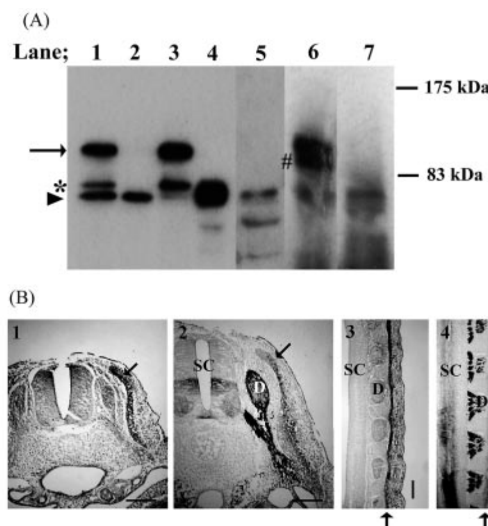

FIG. 6. **The variant and full-length cadherin-7 in chicken embryos.** *A*, immunoblotting and co-immunoprecipitation analysis of fractionated tissue homogenate from E5 chicken embryos, showing the whole homogenate (*lane 1*), soluble fraction (*lane 2*), membranous fraction (*lane 3*), the concentrated conditioning medium of COS variant cells (*lanes 4* and *5*), and immunoprecipitation with anti-variant Ab (*lanes 6* and *7*). The filter was stained with CCD7–1 (*lanes 1–4* and *6*) or anti-variant Ab (*lanes 5* and *7*). The *arrow* indicates full-length cadherin-7; *arrowhead*, the variant protein; *asterisk* and *sharp sign*, degradation products of full-length cadherin-7. *B*, immunostaining. Transverse (*1*, *2*) and horizontal (*3*, *4*) sections of the trunk region in E5 embryos. Whole embryos were stained with anti-variant Ab that detects only the variant form (*1*, *3*), and with CCD7–1 that detects both full-length and the variant form of cadherin-7 (*2*, *4*). *Arrows* indicate dermomyotomes; *SC*, spinal cord; *D*, dorsal root ganglion. *Bars*, 100 μm.

The Variant and Full-length Cadherin-7 Proteins in Chicken Embryos—To localize the variant protein in chicken embryo, we fractionated the homogenate of whole E5 chicken embryos (Fig. 6*A*, *lane 1*) into soluble (Fig. 6*A*, *lane 2*) and membranous fractions (Fig. 6*A*, *lane 3*). CCD7–1 detected three bands in the whole homogenate. The 73-kDa band seemed to be the variant form, because anti-variant Ab detected the 73-kDa band that co-migrates with the variant cadherin-7 expressed in COS variant cells (Fig. 6*A*, *lanes 4* and *5*). The 105- and 80-kDa bands seemed to correspond to the full-length cadherin-7 and its degradation product, respectively (see Fig. 5*C*, *lane 1*). The majority of the variant form was found in the soluble fraction, but some was in the membranous fraction. Because the variant protein was soluble and was still found after resuspension and recentrifugation of the membranous fraction, we believe that the variant form in the membranous fraction interacts with full-length cadherin-7. The full-length and degraded forms of cadherin-7 were found only in the membranous fraction.

To examine the interaction of the variant protein with full-length cadherin-7 *in vivo*, we performed immunoprecipitation from the whole homogenate solubilized with 1% Triton X-100 using anti-variant Ab. Full-length cadherin-7 was co-immunoprecipitated with the variant protein (Fig. 6*A*, *lanes 6* and *7*). These results indicate that some of the variant protein interacts with the full-length cadherin-7. The degradation form of 80 kDa appears to have no interaction with the variant form, because it was not co-immunoprecipitated. The 100-kDa band that was co-immunoprecipitated with anti-variant Ab seemed to be another degradation product of full-length cadherin-7, because this band was often observed for L-cad7 cells (Fig. 6*A*, *lane 6*).

We examined the expression pattern of the variant form in

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E5 chicken embryo tissues, by comparing it with the staining generated by the CCD7–1 antibody that detects both full-length and variant cadherin-7. The variant form was expressed transiently and strongly in dermomyotomes, then at the dorsal and ventral lips and in the myotome. Faint signals of the variant form were detected in proximal dorsal and ventral roots and in dorsal root ganglia (Fig. 6*B*, *1* and *3*). In contrast, the stronger signals of full-length cadherin-7 were detected in dorsal root ganglia and in proximal dorsal and ventral roots, and fainter signals were detected in dermomyotomes (Fig. 6*B*, *2* and *4*). The full-length cadherin-7 was strongly expressed in migrating neural crest cells, but signals of the variant form were faint in these cells (data not shown).

DISCUSSION

In the present study, we first report an identification of the soluble isoform for cadherin-7. Differential expression of the isoform and inhibitory effect on the cell adhesion mediated by full-length cadherin-7 suggest new type of regulatory mechanism of cell adhesion and cell signaling.

Identification of the Soluble Isoform of Cadherin-7—We identified the variant form of cadherin-7 in chicken embryos. Molecular cloning of the genomic fragment revealed that the variant mRNA originated by alternative splicing. Such a splicing variant of type II cadherin has been reported for cadherin-11 (19) and PB-cadherin (20). These variant proteins are truncated forms with an intact transmembrane and a different cytoplasmic domain. The variant protein of cadherin-7 consists of an NH_2-terminal domain, four CRs (CR1–CR4), a shorter pretransmembrane domain, and a distinct COOH-terminal polypeptide of 14 amino acids but lacks transmembrane and cytoplasmic domains. The variant protein might not be a glycosyl phosphatidylinositol-anchored protein, because it does not have the hydrophobic COOH-terminal domain essential for glycosyl phosphatidylinositol-modification (27), in contrast to T-cadherin (6, 7). To investigate whether the variant protein is soluble, we transfected the cDNA of the variant form of cadherin-7 into COS-7 and 293 cells and detected the variant protein in the conditioning medium as a soluble molecule. Furthermore, we fractionated the homogenates of E5 embryos into soluble and membranous fractions. The majority of the variant form was detected in the soluble fraction, and the small amount was found in the membranous fraction. We believe that the variant form of the membranous fraction interacts with full-length cadherin-7. Some soluble forms of the type I and II cadherins that were generated by proteolysis have been reported previously (28, 29). Thus, this is the first demonstration of a soluble form of type I and II cadherins generated by alternative splicing.

Inhibition of Cell Adhesion Mediated by Full-length Cadherin-7 by the Variant Form—It is known that each subtype of cadherin molecules prefers homophilic to heterophilic interactions. Thus, cadherin molecules play a role in intracellular signaling between homotypic cells and in sorting heterotypic cells (5), and the CR1 domain determines their binding specificity (8, 9). The extracellular domain is able to act as a ligand for the same subtype of membranous-bound cadherins and inhibits adhesion mediated by the same subtype of membranous-bound cadherins. The soluble fragment of E-cadherin, generated by proteolysis, is able to disrupt cell-to-cell adhesion in cultured epithelial cells (28) and is involved in carcinoma invasion *in vivo* (30–33). Soluble P-cadherin, generated by proteolysis, is present in human milk and serum of women with or without breast cancer, but the exact function of this protein is not clear (34, 35). And the soluble recombinant T-cadherin inhibits neurite extension from selected neuron populations (36). Several mutations in cadherin-23, a member of the cadherin superfamily, introduce premature stop codons, and the soluble form of cadherin-23, as the result, is produced. These mutations cause hearing loss. The effect of soluble caderin-23 on this symptom is not clear, because it is thought that this arises from a loss of function of full-length cadherin-23 (37, 38).

Both variant and full-length cadherin-7 have the same EC1 domain. It is possible that the variant protein binds to full-length cadherin-7 and inhibits cell-cell adhesion mediated by full-length cadherin-7. To examine this possibility, we performed aggregation assays with or without the variant form. The variant cadherin-7 slowed aggregation, indicating its inhibitory activity against the full-length cadherin-7. Furthermore, to confirm the interaction between the variant protein and the full-length cadherin-7, we performed immunoprecipitation using anti-variant Ab, and full-length cadherin-7 molecules were co-precipitated. Interaction of the variant protein with full-length cadherin-7 suggests that the variant form interrupts trans-interaction of homophilic binding of full-length cadherin-7 *in vitro* and *in vivo*.

Expression and Possible Role of the Variant Form of Cadherin-7 in Chicken Embryos—The expression patterns of cadherin-7 have been well investigated in early chicken embryos (5, 17). The cDNA probe used in these studies for *in situ* hybridization encoded the CR3 and CR4 regions of cadherin-7, and an anti-cadherin-7 monoclonal antibody, CCD7–1, recognized the extracellular domain. Therefore, they detected both full-length and variant forms of cadherin-7 at the transcriptional and protein levels. In this report, we performed competitive PCR and immunostaining using an anti-variant-specific antibody. Full-length cadherin-7 was strongly expressed in migrating neural crest cells; the signal of the variant form was detected strongly on dermomyotomes but only weakly in proximal dorsal and ventral roots and in dorsal root ganglia. The expression pattern of the variant form on dermomyotomes resembles the expression pattern of MyoD at stage 23 (39). The dermomyotome is a developmentally transient tissue that displays extensive cell movements and gives rise to both muscle and dermis.

During development, dynamic cell adhesion, detachment, and migration are essential processes for morphogenesis, and therefore the regulation of cadherin activities must be involved (40). E-cadherin-mediated cell-cell adhesions are dynamically regulated by internal processes. Growth factor activation of receptor tyrosine kinase induces phosphorylation of E-cadherin and β-catenin, ubiquitination, and endocytosis, and epithelial cells then change their morphology (41). Our finding of inhibition against full-length cadherin-7 by a soluble variant form raises the possibility that an alternative regulation mechanism of cadherin activity from outside the cells occurs during development. To establish the mechanism of regulation of cadherin activity by the soluble form, it is necessary to study further *in vivo* on the role of the soluble form in dermomyotome development.

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