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*Original Article*

# Differential expression of neural cell adhesion molecule (NCAM) during osteogenesis and secondary chondrogenesis in the embryonic chick

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**ABSTRACT Progenitor cells in the periosteum-perichondrium of the posterior hook of the quadratojugal (QJ, a membrane bone) in the embryonic chick are bipotential for osteogenesis and chondrogenesis. These cells switch from osteogenesis to chondrogenesis between 10 to 11 days in normal (mobile) embryos but not in paralyzed (immobile) embryos. Expression of neural cell adhesion molecule (NCAM) was studied using a monoclonal antibody in QJ hooks from normal and paralyzed chick embryos between 10 and 21 days of incubation. NCAM is expressed in osteoprogenitor cells and osteoblasts but not in chondroprogenitor cells, chondroblasts, or chondrocytes. The switch of progenitor cell differentiation from an osteogenic to a chondrogenic pathway between 10 and 11 days of incubation coincides with down-regulation of NCAM expression. Both initiation of secondary chondrogenesis and down-regulation of NCAM depend on biomechanical stimulation. In embryos paralyzed at 9 days, secondary cartilage fails to form and progenitor cells remain positive for NCAM. Furthermore, paralysis influences NCAM expression in progenitor cells before secondary chondrogenesis morphologically begins, indicating that NCAM may play a role in the initiation of secondary chondrogenesis. In 15-day normal embryos, NCAM-positive cells accumulate between the perichondrium and secondary cartilage in a position that prevents further cartilage formation in the hook. In 19-day embryos, these cells lose their NCAM expression and restart chondrogenesis in a second phase of differentiation, forming an articular cartilage. Loss of NCAM expression in this cell layer and re-commencement of chondrogenesis do not occur in embryos paralyzed at 13 days, and therefore also require biomechanical stimulation. Hence, down-regulation of NCAM expression correlates with two phases of secondary chondrogenesis in embryonic life, both of which are dependent upon embryonic movement.**

KEY WORDS: *NCAM, cell adhesion molecule, secondary cartilage, chondrogenesis, osteogenesis*

## Introduction

The quadratojugal (QJ), a membrane bone in the upper jaw of birds (Fig. 1A) begins development in 7-day chick embryos as a condensation of neural crest-derived mesenchymal cells. Osteogenesis occurs directly (i.e., without a cartilaginous model) through intramembranous ossification within the condensation, commencing at 7.5 days (Murray, 1963; Hall, 1988). In 10-day embryos, the QJ consists of a shaft and a posterior hook which articulates with the quadrate; both shaft and hook undergo osteogenesis from the periosteum. However, after 10 days, cartilage also develops in the hook (Fig. 1B). Because this cartilage develops from the periosteum, it has been termed secondary (or adventitious) cartilage, together with other similar cartilages in the surangular, pterygoid, squamosal, and palatine (Murray, 1963). Secondary cartilage arises in a periosteum after osteogenesis has commenced (Hall, 1978, 1981; Beresford, 1981), in contrast to primary cartilage which develops directly from a mesenchymal condensation.

Osteogenesis and chondrogenesis in the hook are precisely regulated by biomechanical factors that control the differentiation of progenitor cells in the periosteum-perichondrium. Before 10 days, progenitor cells in the periosteum of the hook and shaft of the QJ undergo only osteogenesis. After 10 days, the progenitor cells in the hook switch from osteogenesis to chondrogenesis and the periosteum of the hook transforms into a perichondrium (Hall, 1972, 1979, 1981; Thorogood, 1979) (Fig. 1B).

*Abbrevations used in this paper:* ABC, avidin-biotin-peroxidase complex; DAB, diaminobenzidine; CAM, cell adhesion molecule; NCAM, neural cell adhesion molecule; PBS, phosphate buffered saline; QJ, quadratojugal.

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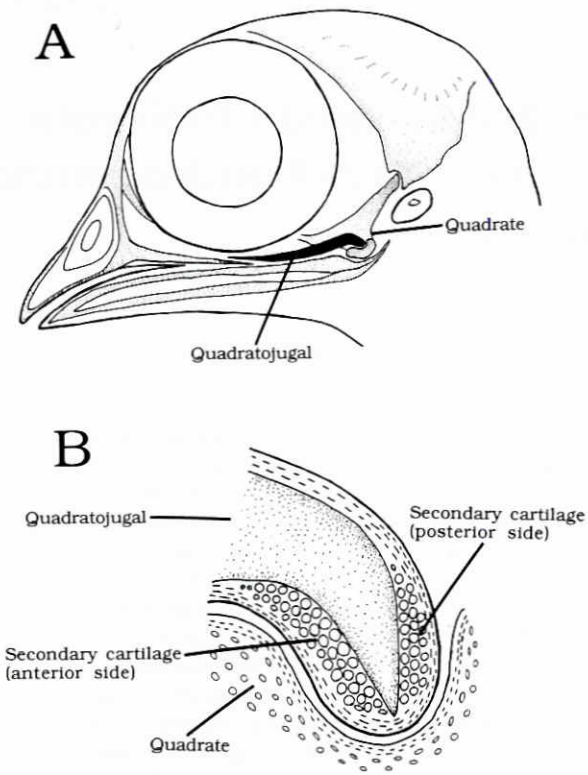



**Fig. 1. Illustrations of a chick head and quadratojugal. (A)** *A lateral view of a chick head showing the position of the quadratojugal (black) in the upper jaw. The bone consists of a shaft and a posterior hook which makes a joint with the quadrate.* **(B)** *A higher magnification of the quadratojugal hook in a 13-day embryo. Secondary cartilage has formed at both anterior and posterior sides of the hook, while osteogenesis continues at the tip.*

Progenitor cels appear to alter their differentiation pathways only in response to local biomechanical stimulation. In normal (mobile) embryos, secondary cartilage forms on the hook. When embryos are paralyzed *before* chondrogenesis is initiated, the progenitor cells in the hook fail to switch to chondrogenesis and continue in osteogenesis (Murray and Smiles, 1965; Hall, 1972, 1979). Therefore, initiation of chondrogenesis at the QJ hook requires biomechanical stimulation.

Although how embryonic movement regulates the differentiation of skeletal progenitor cells is unknown (Hall, 1972, 1979; Hogg and Hosseini, 1992), signal molecules are likely to be required to switch progenitor cells from osteogenesis to chondrogenesis. Embryonic movement may switch on (or off) the expression of signal molecules that regulate the pathway of progenitor cell differentiation. Such a switch may further represent a commitment to secondary chondrogenesis.

Several molecules have been proposed to play regulatory roles in osteogenesis and chondrogenesis. Among them, we are interested in neural cell adhesion molecule (NCAM), a member of the family of cell adhesion molecules (CAMs). Various CAMs have been identified in different tissues and shown to play important roles in cell differentiation, proliferation and morphogenesis (Takeichi, 1988; Linnemann and Bock, 1989; Edelman and Crossin, 1991; Edelman, 1992, 1993). NCAM was the first CAM identified (Jørgensen and Bock, 1974; Brackenbury *et al.*, 1977; Thiery *et al.*, 1977; Jørgensen *et al.*, 1980; Hirn *et al.*, 1981; Noble *et al.*, 1985), and is believed to play an important role in morphogenesis of the nervous system, skin, kidney, muscle (Crossin *et al.*, 1985; Linnemann and Bock, 1989; Knudsen *et al.*, 1990; Soler and Knudsen, 1991; Jiang and Chuong, 1992) and other systems. During chondrogenesis, NCAM is expressed in precartilaginous condensing mesenchyme and in the perichondrium, but is not expressed during differentiation to chondroblasts and chondrocytes (Widelitz *et al.*, 1993; Tavella *et al.*, 1994). Moreover, *in vitro* assays showed that blocking NCAM by antibodies partly inhibited chondrogenesis, while over-expression of NCAM enhanced chondrogenesis (Widelitz *et al.*, 1993). Therefore, NCAM is a possible candidate in regulating cell-to-cell interactions and the initiation of chondrogenesis.

This study examines the process of secondary cartilage formation and NCAM expression during development of the QJ hook in normal and paralyzed embryos between 10 and 21 days of incubation, using a monoclonal antibody against NCAM. Our results show that there are two phases of secondary chondrogenesis in the hook. Changing expression of NCAM correlates with initiation of secondary chondrogenesis, with dedifferentiation of existing secondary cartilage, and with reinitiation of secondary chondrogenesis late in embryonic life. On this basis, we suggest that NCAM is a likely candidate for mediating the effects of embryonic movement and regulating secondary chondrogenesis.

## Results

### *NCAM expression in QJ hooks of 10-day embryos*

In normal 10-day embryos, the QJ consists of bone and surrounding periosteum. No cartilage is present in the QJ hook (Figs. 2A, 5). Immunohistochemistry reveals that NCAM is expressed in the periosteum along the entire shaft and hook (Fig. 2B) and is localized on the cell surface of both progenitor cells and osteoblasts. Young osteocytes are weakly NCAM-positive in their cytoplasm, while old osteocytes are negative.

In embryos paralyzed at 9 days and examined at 10 days, the morphology of the QJ shaft and hook is as in normal, mobile embryos (Fig. 5). No cartilage is found at the hook. The pattern of NCAM expression is similar to that seen in normal embryos. NCAM is uniformly positive in the periostea and osteoblastic cells in all five specimens (not shown).

### *NCAM expression in QJ hooks of 11-day embryos*

QJ hooks in normal 11-day embryos consist of bone but no cartilage (Figs. 3A, 5). In four of five specimens, initiation of secondary chondrogenesis is not found; the morphology of the progenitor cell layer is similar to that seen in 10-day embryos. In the fifth specimen, a few weakly alcian-blue-positive, rounded prechondroblasts were observed at the anterior side of the hook, indicating initiation of chondrogenesis in this specimen. There is no sign of chondrogenesis on the posterior side of the hook in any specimen examined.

NCAM expression changes from 10 to 11 days in the progenitor cells of the hook. The timing of the switch from NCAM-positive to negative differs slightly between the progenitor cells of

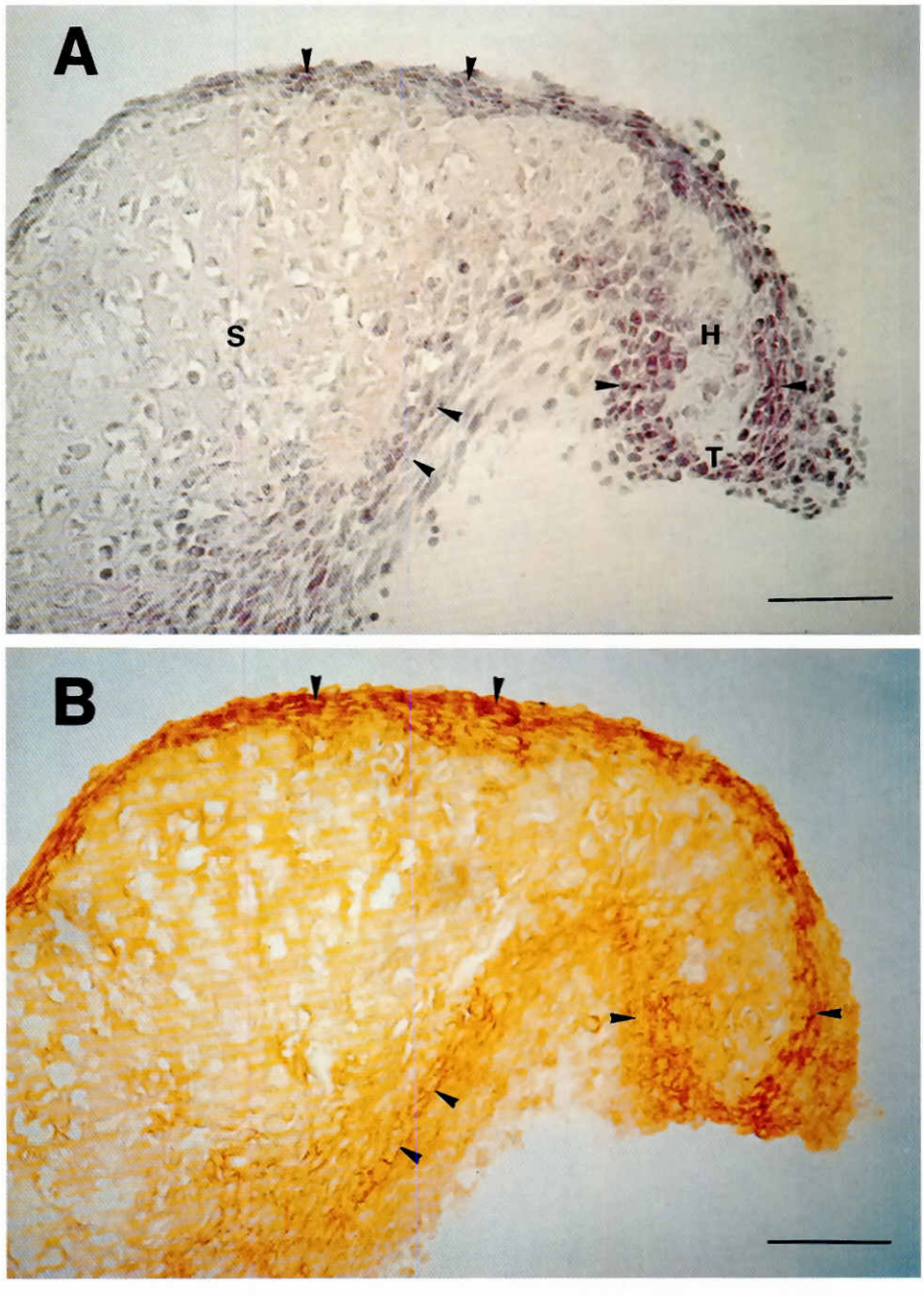



**Fig. 2. NCAM expression in the quadratojugal of a normal 10-day embryo. (A)** *A longitudinal section of the QJ, stained with HBQ to show the shaft (S), hook (H), and tip (T). The periosteum surrounds the entire shaft and hook (arrowheads). No cartilage exists in the hook. Right is posterior, and left is anterior. Bar, 30 μm.* **(B)** *NCAM immunostaining of the section adjacent to that in* **(A)**. *Note that NCAM is localized on the cell surface of the osteoprogenitor cells of both the QJ shaft and hook (arrowheads) as well as of the osteoblasts in the inner side of periosteum.*

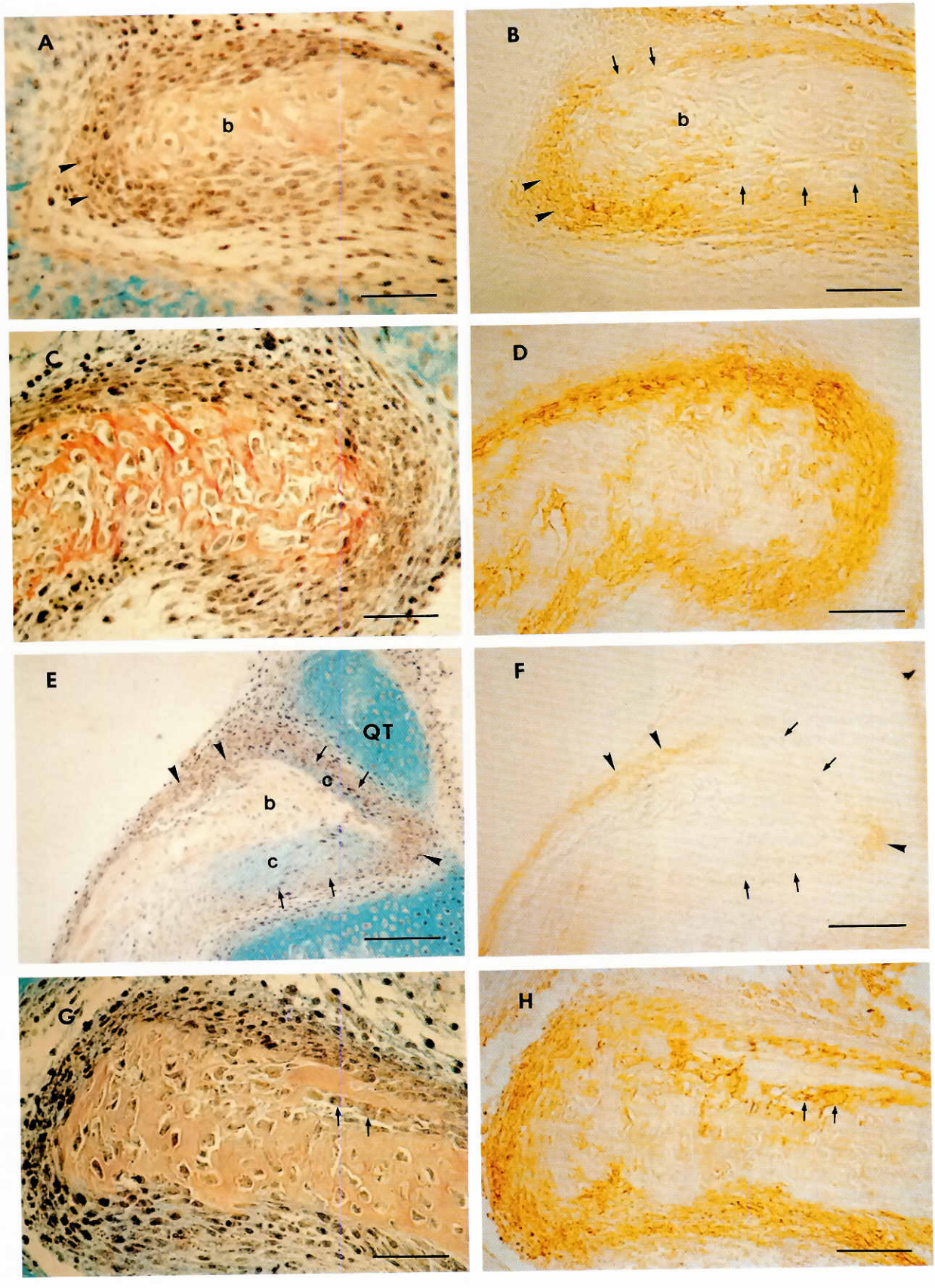
anterior and posterior sides of the hook and between specimens. In the anterior side, most progenitor cells in all specimens have became NCAM-negative (Fig. 3B, arrows), while at the posterior side, only some progenitor cells have switched in some specimens (Fig. 3B, arrows). The tip of the hook remains NCAM-positive (Fig. 3B, arrowheads) and osteogenesis is continuing.

In embryos paralyzed at 9 days and examined at 11 days, the hooks also consist of only bone and surrounding periostea (Figs. 3C, 5). NCAM remains positive in all progenitor cells surrounding the hook (Fig. 3D) and shaft. Paralysis completely prevents the switching off of NCAM expression that occurs in progenitor cells of mobile control embryos.

### *NCAM expression in QJ hooks between 12- and 14-day embryos*

In normal 12-day embryos, a small amount of cartilage tissue is evident in the hooks in all specimens. This cartilage undergoes rapid development. In 13-day embryos, cartilage is very obvious in both sides of the hook, as seen in longitudinal sections after HBQ staining (Figs. 3E, 5). This cartilage does not cover the tip of the hook where bone formation is still continuing (Figs. 3E, 5). The cartilage is larger at 14 days.

In normal mobile embryos between 12 and 14 days, NCAM remains negative in the progenitor cells of the hook except for the tip where osteogenesis is proceeding (Fig. 3F). The cartilage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develops from the NCAM-negative progenitor cells in what is now a perichondrium. All cell types in secondary cartilage — chondroprogenitor cells, chondroblasts, and chondrocytes — as well as their extracellular matrices, are NCAM-negative. At the tip of the hook and along the shaft, osteogenesis still continues and NCAM remains positive in the periosteum. Figure 3F shows the pattern of NCAM expression in the hook in a 13-day embryo.

As the QJ matures, two cell layers can be distinguished in the periosteum of the shaft — an inner, osteogenic cell layer, and an outer, fibroblast-like cell layer. NCAM is strongly positive only in the inner osteogenic cells and very weak or negative in the outer fibroblastic layer (not shown). As blood vessels extend into the bone in the shaft as well as into the bone core in the hook, endosteal ossification begins within the bone. NCAM is expressed in osteoblasts at endosteal ossification sites (not shown).

In embryos paralyzed at 9-days and examined daily up to 14 days, HBQ staining shows that no secondary cartilage has formed in the hook (Figs. 3G, 5), i.e., paralysis completely prevents secondary cartilage formation. NCAM is uniformly positive in the progenitor cells in the hook and along the shaft in paralyzed embryos examined between 12 and 14 days (Fig. 3H). Endosteal ossification can also be seen in the paralyzed embryos as in normal embryos. Osteoblasts in the endosteal ossification sites are also NCAM-positive (Fig. 3H).

### *NCAM expression in QJ hooks in 15- to 17-day embryos*

There are larger pieces of cartilage in both anterior and posterior sides of the hook in normal 15-day embryos. An obvious change at this stage is that a new cell layer (NCL) appears *between* the existing cartilage and the perichondrium (Fig. 4A, arrowheads; Fig. 5, NCL). These cells have some morphological features of chondroblasts, such as a pericellular matrix staining with alcian blue, but have a more rounded shape and a smaller size than regular chondroblasts, indicating that they do not match any single chondrogenic cell type. Such cells appear at the dorsal edge beneath the cartilage earlier in 14-day embryos, and then accumulate along the whole cartilage. In 15-day embryos, these cells cover the entire hook, including the tip, and separate both the preexisting cartilage from its perichondrium and bone from the periosteum at the tip (Figs. 4A, 5). Meanwhile, osteogenesis at the tip ceases. This new cell layer becomes thicker and more obvious in 17-day embryos.

As soon as this cell layer appears, secondary chondrogenesis stops in the hook. Most of the already-formed secondary cartilage becomes an "intermediate tissue" [a tissue with features of both bone and cartilage, as described by Murray (1963) and Hall (1978)] (Figs. 4A; 5, Im). The superficial part of the cartilage close to the new cell layer transforms to bone, especially at the dorsal edge of the cartilage and close to the tip of the hook (Figs. 4A; 5, TB). Immunostaining shows that the new cell layer is NCAM-positive (Fig. 4B, arrowheads), unlike all other chondrogenic cell types; formed cartilage, perichondrium and "intermediate tissue" are all NCAM-negative. Meanwhile, blood vessels invade the hook from the dorsal edge of the secondary cartilage (Fig. 4A, arrows), and multinucleated osteoclasts start to destroy the cartilage, intermediate tissue and bone in the center of the hook.

In embryos paralyzed at 13 days (i.e., after secondary cartilage formation), a new cell layer (with the same morphological features as in normal embryos; Fig. 5) also appears at the hook in 15- and 17-day embryos. Transformation from cartilage to bone is also taking place and may be more rapid in paralyzed embryos than in normal embryos (see below). The new cell layer in the paralyzed embryos is NCAM-positive (not shown), as in normal embryos.

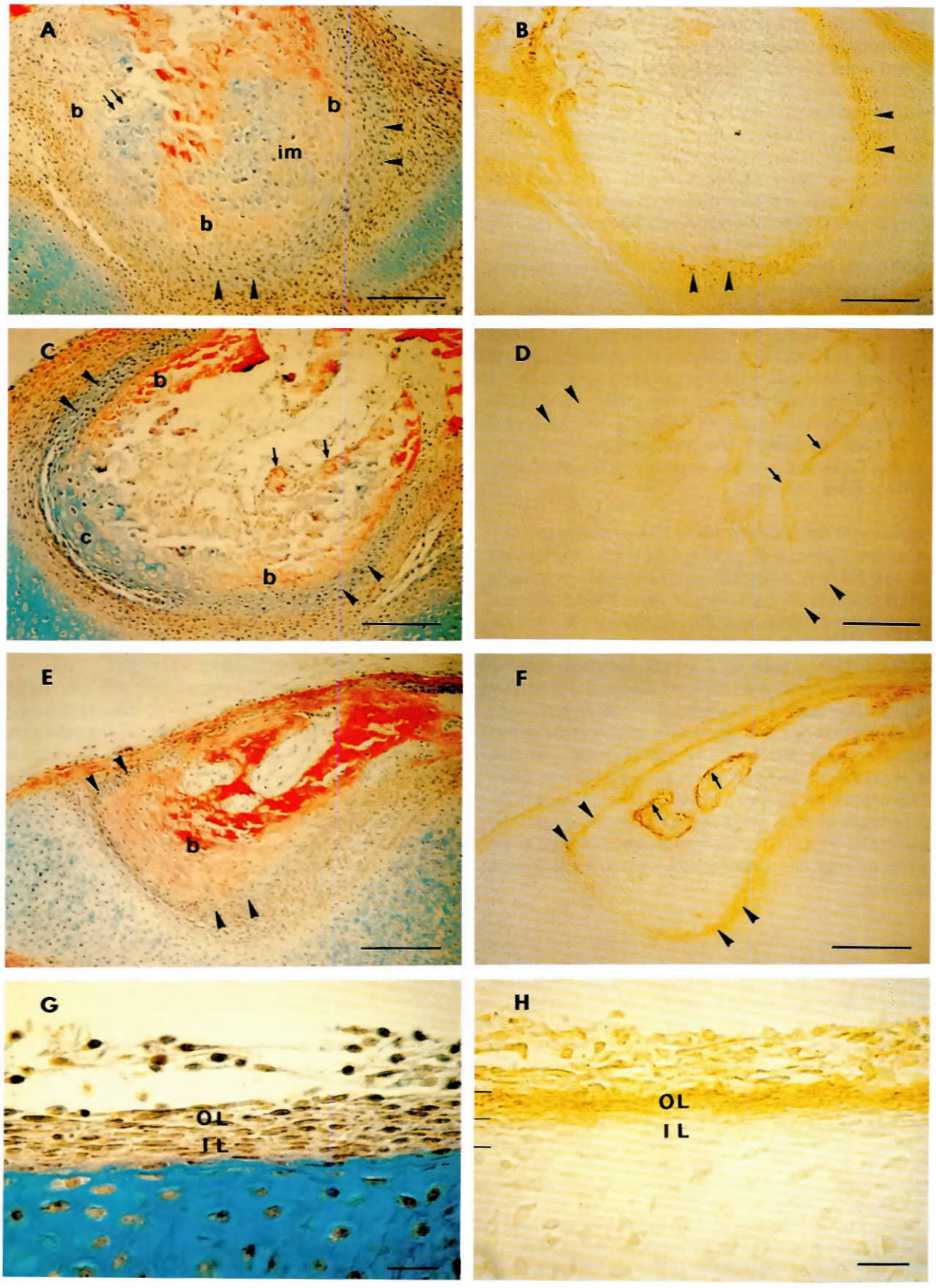
### *NCAM expression in QJ hooks after 19 days*

Most peripheral secondary cartilage has already transformed to bone in the QJ hooks of normal 19-day embryos. In the middle of the hook, intermediate tissue is being destroyed by osteoclasts with ensuing endochondral ossification (Figs. 4C, 5). Meanwhile, some cells in the new cell layer now exhibit the morphological features of chondroblasts and are forming cartilage (Figs. 4C, 5). In 21-day embryos (hatching), the newly-forming cartilage covers the entire QJ hook including the tip, which will serve as an articular cartilage for the quadratojugal-quadrate joint.

Immunohistochemistry shows that the new cell layer has become NCAM-negative in the 19-day embryos (Fig. 4D, arrowheads). Therefore, NCAM expression coincides with a temporary cessation of secondary cartilage formation in this cell layer. When this cell layer begins the second phase of secondary chondrogenesis, NCAM expression is lost. In areas undergoing endochondral osteogenesis, the osteoblasts are NCAM-positive (Fig. 4D, arrows), while osteoclasts are NCAM-negative.

In embryos paralyzed at 13 days (i.e., after cartilage formation) and examined at 19 days, the transformation from secondary

**Fig. 3. Expression of NCAM in the quadratojugal hook of normal embryos and embryos paralyzed before onset of secondary chondrogenesis.** **(A,B).** *NCAM expression in the QJ hook of a normal 11-day embryo.* **(A)** *HBQ staining. There is no morphological sign of secondary chondrogenesis in the QJ hook. Arrowheads indicate the tip of the hook. Top is posterior. b, bone. The cartilage (blue) surrounding the QJ hook is the quadrate. Bar, 20 μm.* **(B)** *NCAM immunostaining of the adjacent sections to* **(A)**. *Most of the progenitor cells at the anterior side of the hook and some at the posterior side have became NCAM negative (arrows), before morphological initiation of secondary chondrogenesis. The cells at the tip are still NCAM positive (arrowheads). b, bone. Bar, 20 μm.* **(C,D)** *NCAM expression in the quadratojugal of a 11-day embryo paralyzed at 9 days.* **(C)** *HBQ staining. Note that the bone core is surrounded by periosteum. Top is posterior. Bar, 20 μm.* **(D)** *NCAM immunostaining of the adjacent section to* **(C).** *Note that the progenitor cells in the hook are NCAM-positive. Paralysis prevents the switch NCAM-positive to negative. Bar, 20 μm.* **(E,F)** *NCAM expression in the quadratojugal of a normal 13-day embryo.* **(E)** *HBQ staining. Note that secondary cartilage (c) has formed on anterior and posterior sides of the bone (b) at the hook. The progenitor cells of both sides are undergoing secondary chondrogenesis (arrows) while osteogenesis continues in progenitor cells in the shaft and at the tip (arrowheads). QT, quadrate. Top is posterior. Bar, 40 μm.* **(F)** *NCAM immunostaining of the section adjacent to* **(E)**. *Note that chondroprogenitor cells, chondroblasts and chondrocytes are NCAM-negative (arrows), while osteoprogenitor cells in the shaft and the tip of the hook remain NCAM-positive (arrowheads). Bar, 40 μm.* **(G,H)** *NCAM expression in the quadratojugal of an embryo paralyzed at 9 days and examined at 13 days.* **(G)** *HBQ staining. The bony core is surrounded by periosteum. Secondary cartilage is absent from the hook. Paralysis completely prevents secondary chondrogenesis. Arrows indicate endosteal osteogenesis. Top is posterior. Bar, 20 μm.* **(H)** *NCAM Immunostaining of the adjacent section to* **(G)**. *NCAM is expressed in the progenitor cells in the hook. Arrows indicate NCAM-positive cells in the area of endosteal osteogenesis. Bar, 20 μm.*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cartilage to bone is faster than in normal embryos; in some specimens, the secondary cartilage has completely transformed into bone (Fig. 4E). The new cell layer mentioned above can still be distinguished (Figs. 4E; 5, arrowheads), but there is no sign of the second phase of chondrogenesis after paralysis. Immunostaining shows that this cell layer remains NCAM-positive in embryos paralyzed at 13 days and fixed at 19 days (Fig. 4F, arrowheads).

### *NCAM expression in the perichondrium of the quadrate*

The perichondrium of the quadrate, a primary cartilage, is thicker than the perichondrium over secondary cartilage in the QJ. Both an inner, progenitor cell layer and an outer, fibroblast-like cell layer can be distinguished (Fig. 4G) in the quadrate. The inner progenitor cell layer is NCAM-negative (Fig. 4H), as is in the perichondrium of secondary cartilage in the QJ. The outer fibroblast cell layer of the quadrate is weakly NCAM-positive (Fig. 4H), although this NCAM-positive layer is absent on the joint surface. Therefore, NCAM expression patterns differ among the perichondrium of a primary cartilage which has negative chondrogenic and weakly positive fibroblastic layers, the perichondrium of secondary cartilage which has only a single negative layer, and the periosteum of bone, which has strongly positive osteogenic inner layer, and a negative fibroblastic outer layer (Table 1).

TABLE 1

**NCAM EXPRESSION IN PERICHONDRIUM AND/OR PERIOSTEUM OF THE QUADRATE (A PRIMARY CARTILAGE), QUADRATOJUGAL HOOK (SECONDARY CARTILAGE) AND QUADRATOJUGAL SHAFT (BONE)a**

| | Outer cell layer | inner cell layer |
|---|---|---|
| Quadrate | + | – |
| Quadratojugal hook | N | – |
| Quadratojugal shaft | – | +++ |

Note: +, +++, intensity of positive NCAM expression; –, NCAM negative; a, all specimens from 13-day embryos; N, no outer cell layer present.

## Discussion

Using a monoclonal antibody we have demonstrated that NCAM is transiently expressed during secondary cartilage development in chicken QJ hooks. Before 10 days, the skeletal progenitor cells in the periosteum of the QJ hook express NCAM and are undergoing osteogenesis. Around 11 days, the progenitor cells in the hook become NCAM-negative and switch from a bone- to a cartilage-formation pathway (although cartilage is not yet seen). NCAM remains negative in all cell types during cartilage development in the hook between 12 and 14 days incubation. Then, a NCAM-positive cell layer accumulates beneath the perichondrium and chondrogenesis ceases in the hook. This cell layer ceases NCAM expression in 19-day embryos and restarts differentiation toward chondrocytes as a second phase of cartilage formation, which is still ongoing at hatching.

NCAM is expressed in osteoprogenitor cells and osteoblasts during periosteal, endosteal and endochondral ossification. Our result is consistent with a previous report of NCAM expression in germinal cells and osteoblasts of tibia, vertebrae and calvaria (Lee and Chuong, 1992). Our use of the ABC method clearly revealed that NCAM is localized on the cell surface of osteoprogenitor cells and osteoblasts. Young osteocytes have weakly-positive cytoplasm, while old osteocytes are completely negative. NCAM expression gradually decreases from osteoprogenitor cells, to osteoblasts, to osteocytes. On the other hand, NCAM is not expressed in any cells associated with chondrogenesis. Therefore, NCAM expression is a feature of osteogenic but not chondrogenic cell lineages.

NCAM expression switches from positive to negative in the progenitor cells of the QJ hook between 10 and 11 days. This change coincides with the timing of commitment for secondary chondrogenesis in the hook (Thorogood, 1979; Hall, 1981) and is earlier than morphological initiation of chondrogenesis. Therefore, NCAM expression seems to be an early marker of the switch from periosteum to perichondrium.

This transient expression of NCAM in the progenitor cells is dependent on mechanical stimulation. In embryos paralyzed before the initiation of secondary chondrogenesis, the progenitor cells continue to express NCAM and their differentiation pathway remains osteogenic. Consequently chondrogenesis is completely prevented in the paralyzed embryos. Therefore, NCAM expression is influenced by embryonic movement, and may provide a molecular mechanism linking embryonic movement to the regulation of secondary chondrogenesis.

**Fig. 4. Expression of NCAM in the quadratojugal hook of normal embryos and embryos paralyzed after onset of secondary chondrogenesis and in the quadrate cartilage. (A,B)** *NCAM expression in the quadratojugal hook of a normal 17-day embryo.* **(A)** *HBQ staining. Secondary cartilage is transforming into intermediate tissue (im) and bone (b). A new cell layer appears between the perichondrium and the secondary cartilage (arrowheads), surrounding the entire hook. Blood vessels are invading the hook (arrows) as endochondral osteogenesis begins. Right is posterior. Bar, 50 μm.* **(B)** *NCAM immunostaining of the section adjacent to* **(A)**. *Note that the new cell layer is NCAM-positive (arrowheads). Bar, 50 μm.* **(C,D)** *NCAM expression in the quadratojugal hook of a normal 19-day embryo.* **(C)** *HBQ staining. Note most intermediate tissue has been destroyed by osteoclasts and endochondral osteogenesis is taking place in the hook (arrows). The new cell layer still exists (arrowheads), but in some area the cells have restarted chondrogenesis so that new cartilage (c) formation can be seen. Left is posterior. Bar, 50 μm.* **(D)** *NCAM immunostaining of the section adjacent to* **(C)**. *Note that the new cell layer has become NCAM-negative (arrowheads). Cells in the areas of endochondral osteogenesis are NCAM-positive (arrows). Bar, 50 μm.* **(E,F)** *NCAM expression in the quadratojugal hook of a 19-day embryo paralyzed at 13 days.* **(E)** *HBQ staining. Secondary cartilage has completely transformed into bone. The hook is smaller than in normal embryos. Note that the new cell layer still surrounds the entire hook (arrowheads). Left is posterior. Bar, 50 μm.* **(F)** *NCAM immunostaining of the section adjacent to* **(E)**. *Note that the new cell layer remains NCAM-positive (arrowheads). Bar, 50 μm.* **(G,H)** *NCAM expression in the perichondrium of the quadrate, a primary cartilage.* **(G)** *HBQ staining of the perichondrium. An outer fibroblast-like cell layer (OL) and an inner progenitor cell layer (IL) can be distinguished. Bar, 10 μm.* **(H)** *NCAM immunostaining of the adjacent section to* **(G)**. *The outer fibroblastic cell layer (OL) of the perichondrium is NCAM-positive, the inner progenitor cell layer (IL) is NCAM-negative, as are chondroblasts and chondrocytes. Bar, 10 μm.*

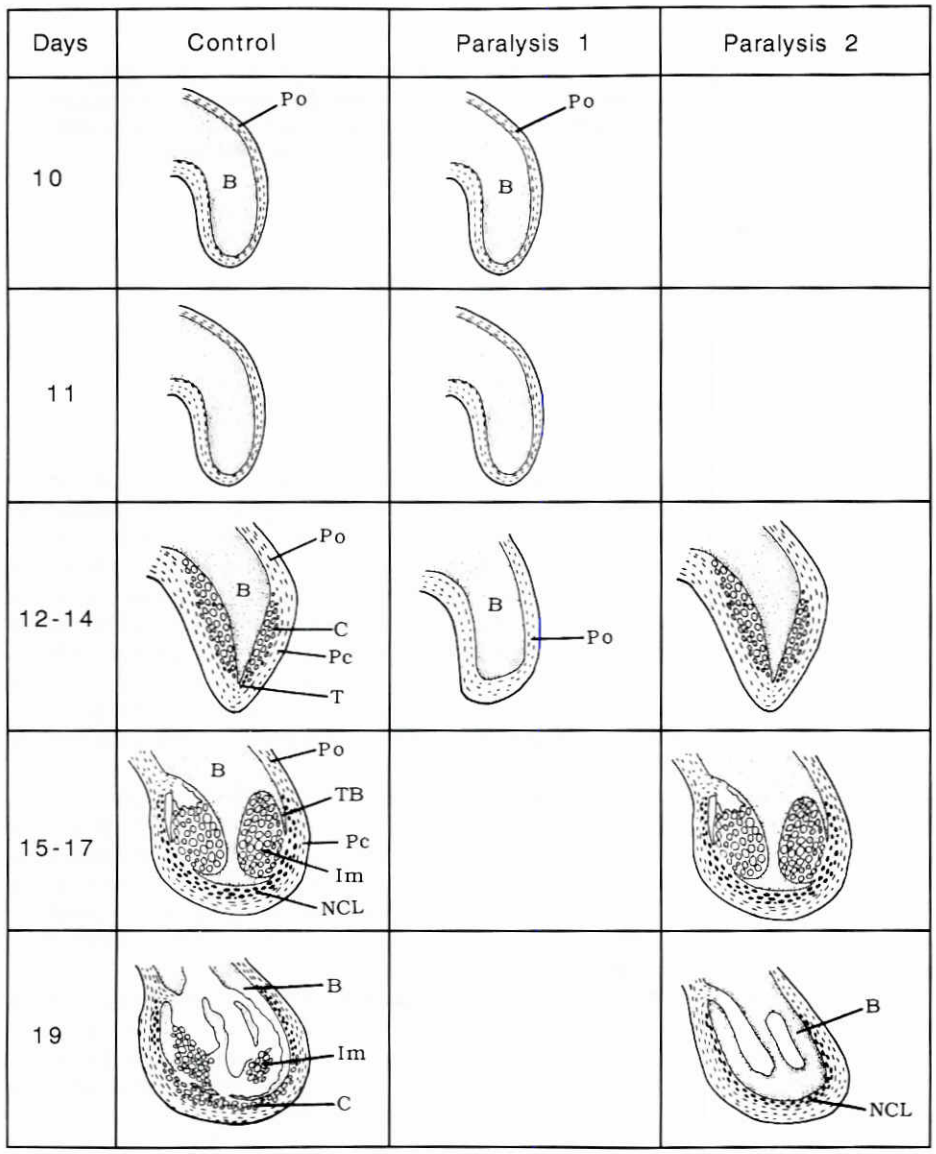


**Fig. 5. Illustration of morphological development of the QJ hook in control (normal embryos), paralysis 1 (paralyzed before cartilage formation), and paralysis 2 (paralyzed after cartilage formation). (Control)** *There is no morphological change between 10 and 11 days. At 12 to 14 days, secondary cartilage develops in the hook except for the tip where osteogenesis continues. At 15 to 17 days, a new cell layer (NCL) appears between the cartilage and perichondrium. Chondrogenesis ceases and the already-formed cartilage either becomes intermediate tissue (Im) or transforms into bone (TB). By 19 days, the NCL restarts chondrogenesis and new cartilage (C) forms at the surface of the hook. Intermediate tissue is being destroyed by endochondral ossification. Po, periosteum. Pc, perichondrium. B, bone. T, tip.* **(Paralysis 1)** *No cartilage formation is found in the hooks in 12- to 14-day embryos. Paralysis completely prevents secondary cartilage formation in the hook.* **(Paralysis 2)** *From 12 to 17 days, the hooks have the similar morphology as normal embryos. However, at 19 days, the NCL still remains and there is no second phase of cartilage formation in the hook. Endochondral ossification in the hook is more rapid than in normal embryos.*

A cascade of genes are likely to be turned on or off as progenitor cells change their pathway from osteogenesis to chondrogenesis in the QJ hook. Among them, one gene (or molecule) may act as a switch regulating this change. Altering expression of this switch gene should correlate with the timing of commitment for secondary chondrogenesis (between 10 and 11 days) and embryonic movement. Since the change of NCAM expression in QJ hook just precedes this commitment and requires embryonic movement, NCAM satisfies some requirements for this switching molecule and, therefore, may play a role in controlling the alternative pathways of osteo- and chondrogenesis in the progenitor cells.

It has been reported that over-expression of NCAM enhances chondrogenesis while exposure to NCAM antibody partly inhibits chondrogenesis in limb bud mesenchymal cells *in vitro* (Chuong *et al.*, 1993; Widelitz *et al.*, 1993). NCAM expression, therefore, is required for chondrogenesis in mesenchymal cells. However, our study demonstrates that NCAM expression is required by osteogenesis but not by chondrogenesis in secondary cartilage formation. It should be noted that secondary chondrogenesis differs from chondrogenesis in mesenchyme. In order to initiate chondrogenesis, mesenchymal cells have to undergo condensation (Hall and Miyake, 1992), and NCAM expression is necessary for mesenchymal cells to condense (Widelitz *et al.*, 1993). However, the QJ is a membrane bone. The progenitor cells in the periosteum at 10 days have passed the condensation stage (Murray, 1963) and are bipotential for osteogenesis and chondrogenesis. According to our observations, NCAM expression is up-regulated when these progenitor cells undergo osteogenesis, and down-regulated when they undergo chondrogenesis.

An unusual finding is that, after secondary cartilage is well developed in the hook, a new NCAM-positive cell layer appears between the perichondrium and secondary cartilage at 15-17 days. That this cell layer has not been noted before is probably because these cells have some morphological features of early

chondroblasts. However they are NCAM-positive and so obviously differ from the cell types involving in cartilage formation. The position and morphology of these cells suggest that they dedifferentiated from chondroblasts. As soon as this NCAM-positive cell layer appears, the secondary cartilage deep to this cell layer transforms into an intermediate tissue in which endochondral ossification is initiated. Hence, on the basis of these observations, it is possible that the new cell layer may regulate the cessation of chondrogenesis and remodeling of the formed cartilage in the hook. Interestingly, this new cell layer stops NCAM expression and restarts chondrogenesis in 19-day embryos. This switch from NCAM-positive to negative and reinitiation of chondrogenesis depends on embryonic movement as does initial formation of secondary cartilage. This raised the question of whether NCAM also plays a role in controlling the cessation and subsequent reinitiation of chondrogenesis in this new cell layer.

## Materials and Methods

### *Incubation of chick embryos*

Fertilized white leghorn chicken eggs were obtained from Cook's Hatchery, Truro, Nova Scotia, Canada and incubated in a forced-draft Humidaire incubator at a temperature of 36±0.5°C. Quadratojugals from embryos of 10 to 15, 17, 19 and 21 days of incubation were used for the study.

### *Paralysis of chick embryos*

Paralysis was carried out by injecting decamethonium iodide (Koch-Light Labs Ltd., Colnbrook, UK) dissolved in sterile saline (0.85% NaCl) into the air sac of embryonated eggs. Before injecting, the surface of the egg was swabbed with 70% ethanol and a pinhole was made in the shell at the edge of the air sac. Decamethonium iodide was injected through the pinhole with a sterile syringe, the pinholes were sealed with Scotch tape and the eggs were returned to the incubator for further incubation. The effectiveness of paralysis can be judged by observing embryonic movement when opening the eggs. Two experimental groups were established, varying in the time of paralysis and fixation.

*Paralysis of embryos before cartilage formation*

Embryos were paralyzed by injecting 1.0 mg decamethonium iodide (0.5 ml of a 2 mg/ml solution) into each egg at 9 days of incubation. 9 day is the time before initiation of secondary chondrogenesis (Murray, 1963; Hall, 1972). Embryos of the same stage were injected with PBS as control. The quadratojugals and associated parts of the quadrates from 10- to 15-day embryos were removed and fixed for histology and immunohistochemistry.

*Paralysis of embryos after cartilage formation*

1.75 mg of decamethonium iodide (0.5 ml of a 3.5 mg/ml solution) was injected into each egg at 13 days of incubation. By 13 days secondary cartilage is well developed in the QJ hook. Embryos of the same stage were injected with PBS as control. The QJs and parts of the quadrates from 15-, 17-, and 19-day embryos were removed and fixed for histology and immunohistochemistry. With this dose, about half the embryos survived for 48 h, and about 25% survived for 6 days. With lower doses, embryos could not be paralyzed reliably, while higher doses were lethal.

### *Preparation of specimens for immunohistochemistry and histology*

The QJs and parts of the quadrates from at least five embryos per day for each of the control and experimental groups were fixed in periodate-lysine-paraformaldehyde (PLP) fixative overnight (McLean and Nakane, 1974). The tissues were dehydrated in ethanol, infiltrated and embedded in low melting point paraffin at 52°C, and serial sections were cut with a microtome at 5 μm. To increase adhesion, sections were mounted on poly-L-lysine-coated slides.

### *Antibody and reagents*

Monoclonal antibody against NCAM was obtained from the Developmental Studies Hybridoma Bank. This antibody (5e) which was developed with chicken brain NCAM as immunogen, recognizes the extracellular domain of 120, 140 and 180 kD NCAM polypeptides (Watanabe *et al.*, 1986). Biotinylated goat anti-mouse IgG was purchased from Gibco BRL, Canada. Avidin-biotin-peroxidase complex kit (VECTASTAIN elite ABC kit PK-6100) and normal goat serum were obtained from Vector Laboratories (Buringame, CA, USA). Hydrogen peroxide ($H_2O_2$) and diaminobenzadine (DAB) were purchased from Sigma (St. Louis, MO, USA).

### *Immunohistochemistry*

The Avidin-biotin-peroxidase complex (ABC) immunohistochemical technique was modified from Hsu *et al.* (1981). Sections were deparaffinized in histoclear and taken through graded ethanols into distilled water. Then they were rinsed with PBS containing 0.25% triton X 100 (as in the following rinsing) and blocked with 100% normal goat serum for 20 min at room temperature. After draining, sections were incubated with 1:10 anti-NCAM supernatant diluted with PBS containing 10% normal goat serum for 2 h at room temperature or overnight in 4°C. Sections were rinsed and then incubated with biotinylated goat anti-mouse IgG diluted 1:200 with PBS containing 10% normal goat serum for 30 minutes at room temperature. After rinsing with PBS, the sections were loaded with Avidin-biotin-peroxidase complex (ABC) for 30 min at room temperature. The sections were then rinsed with PBS and a brown precipitate was produced by treating the specimens with 3,3'-diaminobenzidine (DAB) [1 DAB tablet (10 mg) was dissolved in 20 ml 0.05M, pH 7.6 Tris-HCl buffer containing 4 μl 30% hydrogen peroxide ($H_2O_2$)] for 1.5-2 minutes at room temperature. As a negative control, the primary antibody was replaced with PBS — no non-specific reaction was observed. To check for endogenous peroxidase, some sections were treated with the ABC and DAB steps. No endogenous peroxidase reaction was found in the specimens.

Adjacent sections to those processed for immunohistochemistry were stained with Hall and Brunt's quadruple (HBQ) staining (Hall, 1986). This method uses celestial blue B, alcian blue, Mayer's hematoxylin, and direct red, and gives high contrast between cartilage (blue) and bone (red).

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