Formation of Cartilage in the Heart of the Spanish Terrapin, *Mauremys leprosa* (Reptilia, Chelonia)

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**ABSTRACT** Cartilaginous deposits are regularly present in the heart of several reptilian, avian, and mammalian species. The formation of these extraskeletal cartilages has been studied in birds and mammals, but not in reptiles. The aim here was to elucidate this question in the Spanish terrapin. Hearts from 23 embryos belonging to Yntema (1968) developmental stages 17 to 26 and eight terrapins age 3 months to 10 years were examined using histological, histochemical, and immunohistochemical techniques. In the heart of the Spanish terrapin (*Mauremys leprosa*), chondrogenesis can start during embryonic life. Cartilaginous tissue develops from a mesenchymal cellular condensation that extends along the aortico-pulmonary septum and the incipient pars fibrosa of the ventricular horizontal septum. This cellular condensation, which is smooth muscle α-actin (SMα-actin)-negative and type II collagen-negative during stages 17 to 22, acts as a prechondrogenic condensation. In stage 23, production of type II collagen begins in the central core of the condensation and gradually spreads toward its periphery. The type II collagen-positive (chondrogenic) cellular condensation remains devoid of perichondrium prior to birth. Thereafter, it converts into hyaline cartilage that extends along the proximal part of the aortico-pulmonary septum and the pars fibrosa of the horizontal septum. Our findings are consistent with the assumption that, as in birds and mammals, the precursors of the cardiac chondrocytes are cells originating from the neural crest. In birds, the development of cardiac cartilage takes place during embryonic life and is initiated with the formation of mesenchymal cell condensations, whereas in the mammalian heart chondrogenesis starts after birth without formation of conspicuous prechondrogenic condensations.

The present work was designed to illustrate the formation of cartilaginous tissue in the heart of the Spanish terrapin, *Mauremys leprosa*, a living representative of the anapsid reptiles. The ultimate goals of our study were 1) to gain new insight into the ontogeny of the cardiac cartilages in amniotes from 1956, 1959; Webb, 1979; Young, 1994, for original descriptions and extensive reviews of the literature. This early work is concerned with the histological features, shape, size, location, and function of the cartilages occurring in adult animals. The hyaline cartilaginous deposits vary in shape and size. In turtles, rynchchocephalians, lizards, and snakes the deposits are usually located in portions of the cardiac outflow tract, such as the aortico-pulmonary and interaortic septa. In crocodiles the cartilage appears in the interventricular septum and in the semilunar and atrioventricular valves.

**KEY WORDS:** cartilage; heart, embryo; reptilia; chelonia

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Cartilaginous deposits are regularly present in the cardiac fibrous skeleton of several reptilian species (see Matumoto, 1938; Kashyap, 1950; White, 1956, 1959; Webb, 1979; Young, 1994, for original descriptions and extensive reviews of the literature). This early work is concerned with the histological features, shape, size, location, and function of the cartilages occurring in adult animals. The hyaline cartilaginous deposits vary in shape and size. In turtles, rynchchocephalians, lizards, and snakes the deposits are usually located in portions of the cardiac outflow tract, such as the aortico-pulmonary and interaortic septa. In crocodiles the cartilage appears in the interventricular septum and in the semilunar and atrioventricular valves.

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a comparative viewpoint, and 2) to gather new data on the possible morpho-functional significance of such cartilages. The study was carried out in embryonic and adult hearts from Spanish terrapins, which were examined using histological, histochemical, and immunohistochemical techniques.

MATERIALS AND METHODS

Animals

Fertilized eggs were obtained from adult females of the Spanish terrapin, Mauremys leprosa (Schweigger, 1812), collected in the environs of Badajoz, Extremadura, Spain. The animals were kept in terraria, where food was given as required. Oviposition was induced by injecting oxytocin into females (1 IU/100 g of body weight). The eggs were kept in a rocking incubator at 28°C in a 90% humid environment. At these conditions, the average time of incubation is about 79 days. Twenty-three embryos were collected between 22 and 79 days of incubation. The formation of cartilaginous tissue in embryonic hearts was correlated to the developmental stages established by Yntema (1968) in the common snapping turtle, Chelydra serpentina. It should be noted that these stages have previously been used successfully for the Spanish terrapin (Kalman et al., 1997). Table 1 shows the distribution of the embryos examined, according to the developmental stages.

In addition, the hearts from eight specimens, ages 3 months to 10 years, were studied: 3 months (n = 2), 5 years (n = 1), 4 years (n = 2), 5 years (n = 1), 6 years (n = 1), and 10 years (n = 2). These specimens had been collected in the environs of Badajoz and used in previous anatomical studies. Their respective ages were estimated according to the number of growth lines present in the horny scutes of the carapace (Castanet and Cheylan, 1979).

All the animals were collected and sacrificed with permission (VS 99/198; AFG/afg) of the Regional Government of Extremadura, Spain, and were handled in accordance with the Spanish Regulations for the Protection of Experimental Animals (Real Decreto 223/1988).

The hearts of 22 embryos, two young animals (3 and 18 months), and three adults (4, 5, and 10 years old) were examined by histological, histochemical, and immunohistochemical techniques for light microscopy. The heart of the remaining embryo (developmental stage Y25) and the outflow tract of the three remaining adults were stained in toto with Alcian blue.

| Table 1. Embryos examined, according to the developmental stages of Yntema (1968) |
|---|---|
| dps | n | di |
| 17 | 1 | 22 |
| 18 | 1 | 25 |
| 19 | 2 | 29 |
| 20 | 2 | 32 |
| 21 | 2 | 34 |
| 22 | 2 | 37 |
| 23 | 3 | 38–47 |
| 24 | 4 | 48–56 |
| 25 | 5 | 58–70 |
| 26 | 1 | 70–80 |

Abbreviations: di, days of incubation; dps, developmental stages; n, number of specimens examined.

Histological and Histochemical Techniques

Whole embryos or isolated hearts were fixed by immersion in Bouin’s fluid (ratio of fixative to tissue volume = 80:1) and embedded in Paraplast (Sigma Chemical Co., UK). Serial sections, cut transversely, longitudinally, or obliquely at 10 μm, were stained with hematoxylin-eosin or Mallory’s trichrome stain for a general assessment of the histological components of the heart. Other procedures used were Weigert’s resorcin-fuchsin for staining elastin and the von Kossa technique (Kiernan, 1990) for the specific detection of calcium deposits in cardiac cartilages of adult animals. Sections were examined with a light microscope and photographed with a photomicrographic attachment.

Immunohistochemical Techniques

Whole embryos or isolated hearts were fixed by immersion in Bouin’s fluid, embedded in Paraplast, as described above, and cut transversely or longitudinally at 10 μm. Sections were stained with monoclonal antibody CIIC1 (Developmental Studies Hybridoma Bank, University of Iowa, Ames, Iowa) recognizing type II collagen, or with monoclonal anti-SMa-actin (Sigma, clone 1A4).

The use of the immunohistochemical technique for the detection of type II collagen relied on the fact that synthesis of type II collagen is considered cartilage-characteristic (Miller and Matukas, 1968; Miller, 1976; Kosher, 1983; Hall and Miyake, 1992,1995), even though type II collagen is also produced by a limited number of nonchondrogenic cell types (see Kosher, 1983, and Swiderski et al., 1994, for extensive reviews of the literature). The anti-SMa-actin was utilized bearing in mind that in the chick and quail the use of this antibody was conclusive in deciding the nonmuscular nature of the neural-crest derived cells which are believed to be the precursors of the cardiac chondrocytes (López et al., 2000).

The sections were dewaxed in xylene, hydrated in an ethanol series, and washed in Tris-phosphate buffered saline (TPBS, pH 7.8). For the detection of type II collagen, the tissues were then digested for 15–30 min at 37°C with 0.5% papain in PB (pH 4.7). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in TPBS for 30 min. After washing with TPBS, nonspecific binding sites were saturated for 30 min with 10% sheep serum and 1% bovine serum albumin in TPBS (SB) for staining with CIIC1, or with the same solution plus 0.5% Triton X-100 (SBT) for staining with anti-SMa-actin. Sections were then incubated overnight at 4°C in the primary antibody diluted in SB when staining with CIIC1 or in SBT when staining with anti-SMa-actin. Control slides were incubated in SB or SBT only. After incubation, the sections were washed in TPBS (3 × 5 min), incubated for 1 h at room temperature in biotin conjugated antismouse IgG (Sigma) diluted 1:100 in TPBS, washed again, and incubated for 1 h in ExtrAvidin® conjugate (Sigma) diluted 1:150 in TPBS. Peroxidase activity was developed with Sigma Fast® 3,3′-diaminobenzidine tablets according to the indications of the supplier. In several cases the sections were counterstained with hematoxylin or hematoxylin-eosin.

Alcian Blue In Toto Staining Technique

The heart was removed, transferred to Ringer’s solution, and dissected to expose the cardiac outflow tract. The specimens were fixed by immersion in 5% trichloroacetic acid (ratio of fixative to tissue volume = 80:1) and stained with Alcian blue 8 GX (Gurr), according to the technique described by Ojeda et al. (1970), then rinsed with 70% aqueous ethanol.

Nomenclature

The terms proximal and distal are used to describe the location of the cardiac outflow tract components with regard to the ventricle, while anterior and posterior are employed to indicate the situation of the ventricular components with regard to the anteroposterior axis of the heart.

RESULTS

The ventricle of the chelonian heart is divided into a large cavum dorsale and a smaller cavum ventrale,
or cavum pulmonale, by an incomplete horizontal septum, also called muscular ridge, Muskelleiste, or interventricular septum (Greil, 1903; Goodrich, 1919,1930; Leene and Vorstmann, 1930; Kashyap, 1959; Guibé, 1970; Webb et al., 1974; Holmes, 1975; Webb, 1979; Van Mierop and Kutsche, 1981,1985). In several species a variably developed vertical septum usually subdivides the cavum dorsale into the cavum venosum and cavum arteriosum. In the Spanish terrapin, the horizontal septum is the major septal structure within the ventricle. The vertical septum is so poorly developed as to be practically indistinguishable from other muscular trabeculae. As in other turtles, the horizontal septum consists of two portions: a posterior (apical) complete portion and an anterior incomplete portion. The posterior portion is of a muscular nature and separates the cavum dorsale from the cavum ventrale. The anterior portion assumes a subhorizontal position; its dorsolateral margin is free, allowing communication between the dorsal and ventral chambers. The free margin is covered by a thick layer of fibrous tissue, which we call the pars fibrosa of the horizontal septum. This pars fibrosa connects with the aorticopulmonary septum, a fact that needs to be emphasized, since the largest cartilaginous focus observed in the heart of the Spanish terrapin develops inside these two anatomical elements of the cardiac outflow tract (Fig. 1).

Embryological Findings

In the earliest embryo examined (stage Y17), the aorticopulmonary and interaortic septa were fully developed. In this specimen, as well as in the embryos of developmental stages 18 to 22, a conspicuous condensation of mesenchymal cells extended along the central part of the aorticopulmonary septum, penetrating the incipient pars fibrosa of the horizontal septum (Figs. 1, 2A,B). The portion of the cellular condensation corresponding to the aorticopulmonary septum was SMα-actin-negative and type II collagen-negative. In the Y22 embryos, the condensation clearly stood out against the surrounding medial tissue of the great arteries, which had acquired an organization of SMα-actin-positive lamellar cells and SMα-actin-negative interlamellar cells (Fig. 3).

The portion of the cellular condensation located in the anticipated pars fibrosa of the ventricular horizontal septum also displayed a type II collagen-negative extracellular matrix. Yet some scattered SMα-actin-positive cells were present in this part of the condensation (Figs. 1, 4). The highest level of SMα-actin immunoreactivity was recorded in embryos belonging to stage 22 at the caudal end of the condensation, that is, at the boundary between the anticipated pars fibrosa and the myocardial tissue of the horizontal septum (Figs. 1, 5). The SMα-actin expression quickly disappeared from this site in subsequent developmental stages (Y23–Y24).

Two of the four Y23 embryos showed a condition similar to that of the preceding developmental stages. In the other two, type II collagen was present in the extracellular matrix of the central core of the cellular condensation. This occurred along the portion of the condensation located between the proximal part of the aorticopulmonary septum and the anticipated pars fibrosa of the ventricular horizontal septum (Figs. 1, 6). In the Y24 to Y26 embryos, the production of type II collagen had increased toward the periphery of the condensation (Fig. 7).

Cardiac Cartilage in Young and Adult Animals

In the young terrapin, age 3 months, there was a well-developed cartilaginous deposit, which extended along the proximal part of the aorticopulmonary septum and pars fibrosa of the horizontal septum. The cartilage appeared as an elongated bar and was hyaline. The chondrocytes were embedded in a type II collagen-positive cellular matrix (Fig. 8). The deposit was surrounded by a thin perichondrium.
In the other young animal, age 18 months, and in all six adult terrapins, 4–10 years old, there were two cartilaginous deposits in the heart. One of them was located in the pars fibrosa of the horizontal septum and proximal portion of the aorticopulmonary septum, reaching the proximal level of the interaortic septum (Figs. 1, 10A). Histological sections revealed that the cartilage was hyaline, with small, spheroidal chondrocytes (Fig. 10B). The perichondrium was thin, sometimes even incomplete. At the sites that were devoid of perichondrium the cartilaginous tissue merged gradually with the surrounding connective tissue.

The other cardiac deposit extended along the sinus wall of the right semilunar valve of the right aorta (Figs. 1, 10A), penetrating the fibrous cushion that constitutes the proximal support of the corresponding valve leaflet (cusp). In the 18-month terrapin, this second focus consisted of a small group of chondrocytes, which were embedded in a type II collagen-positive cellular matrix that stained markedly with hematoxylin and was devoid of perichondrium. In the adult animals, the focus was of hyaline cartilaginous tissue and was surrounded by a thin perichondrium.

Finally, it should be noted that the cartilaginous deposits of the adult animals displayed no hypertrophied chondrocytes, nor mineralization of the matrix.

**DISCUSSION**

In 1938, Matumoto stated that in reptiles the formation of cardiac cartilage is initiated after birth. He based his conclusion on the fact that he detected no cartilaginous focus in embryonic hearts of the gekkonid *Hemidactylus bowringii* and the viperid *Gloydius blomhoffii*, referred to as *Ancistrodon blomhoffii*. Subsequently, Van Mierop and Kutsche (1985) mentioned the occurrence of cartilage in the base of the heart and near the valves in an alligator embryo about half-way through incubation (see the discussion following the oral presentation of the contribution by the authors cited). To our knowledge, no other data concerning the ontogeny of cardiac cartilage in reptiles have been reported.

Our observations demonstrate that in the chelonian heart chondrogenesis can start during embryonic life. In the Spanish terrapin, synthesis of type II collagen, which can be considered the first unequivocal sign of cartilage formation, begins as early as stage Y23 (38–47 days of incubation). This occurs in the core of the mesenchymal cellular condensation that extends along the aorticopulmonary septum and incipient pars fibrosa of the ventricular horizontal septum. Therefore, this mesenchymal condensation acts as a prechondrogenic condensation. The
differentiation of the mesenchymal cells into chondrocytes seems to proceed from the center of the condensation to its periphery. The type II collagen-positive cellular condensation remains devoid of perichondrium prior to birth. Thereafter, it becomes gradually converted into hyaline cartilage.

The chondrogenetic process occurring in the heart of the Spanish terrapin is very similar to that which takes place in the aortic and pulmonary valves of birds. In these valves, formation of cartilaginous tissue also begins during embryonic life (Stiefele, 1926; Matumoto, 1938; Tsusaki et al., 1956; Sumida et al., 1989; López et al., 2000). Chondrogenesis starts with the formation of conspicuous prechondrogenic condensations, which consist of loosely packed mesenchymal cells embedded in a type II collagen-negative extracellular matrix (López et al., 2001). Then the prechondrogenic condensations differentiate into chondrogenic (type II collagen-positive) condensations, which become transformed into hyaline cartilage after hatching. In mammals, the formation of cartilage in the cardiac semilunar valves is somewhat different. It starts within the first month of life, and does not involve formation of prechondrogenic condensations (López et al., 2001). The cartilaginous foci occurring in the aortic and pulmonary valves of birds are believed to originate from neural crest-derived cells (Sumida et al., 1989; Bachnou et al., 1996) of a nonmuscular nature (López et al., 2000), which invade the cardiac outflow tract during embryonic life (Takamura, 1990; Yablonka-Reuveni et al., 1995,1998; Bergwerff et al., 1996,1998; Poelmann et al., 1998; Waldo et al., 1998,1999). The cardiac chondrocytes in mammals are also presumed to derive from neural crest cells (López et al., 2001) occurring in the fibrous skeleton of the heart during both embryonic (Waldo et al., 1999; Jiang et al., 2000) and postnatal life (Jiang et al., 2000). The contribution of the neural crest to the configuration of the cardiac outflow tract in reptiles is still uncertain. Nonetheless, it seems plausible to assume that, as in birds and mammals (Poelmann et al., 1998; Waldo et al., 1998; Jiang et al., 2000), cells from the cardiac neural crest may play a decisive role in the septation of the embryonic conotruncus in reptiles, contributing to the formation of the aortico-pulmonary septum. Indirect evidence supporting this suggestion is provided by the fact that the mesenchymal cellular condensation that extends along the developing aortico-pulmonary septum of the Spanish terrapin is anatomically similar to that of neural crest-derived cells occurring in birds (Takamura, 1990; Yablonka-Reuveni et al., 1995,1998; Bergwerff et al., 1996,1998; Poelmann et al., 1998; Waldo et al., 1998,1999) and mammals (Waldo et al., 1999; Jiang et al., 2000). Therefore, we presume that, as in these two groups, the precursors of the cardiac chondroblasts in the Spanish terrapin are neural crest-derived elements. Moreover, the SMα-actin-negative condition of these neural crest elements in the embryos examined is consistent with the assumption that they are of a nonmuscular nature.

An interesting point is that, prior to the synthesis of type II collagen in the portion of the cellular condensation located in the pars fibrosa of the ventricular horizontal septum, several cells of the condensation are transiently SMα-actin-positive. SMα-actin expression is usually associated with the differentiation of mesenchymal cells into smooth muscle cells. However, it is known that transient expression of SMα-actin may indicate cell migration (Waller et al., 2000). Bearing in mind that in the adult Spanish terrapin the pars fibrosa of the ventricular horizontal septum is devoid of smooth muscle cells, cell migration seems to be the only explanation for the SMα-actin immunoreactivity detected at this site. This assumption, together with the presumed neural crest origin of the condensed cells in the pars fibrosa, leads to the following suggestion: In the Spanish terrapin, cells from the neural crest may populate the embryonic pars fibrosa of the horizontal septum, thereby contributing to its alignment with the aorticopulmonary septum. Further studies using appropriate techniques to detect neural crest cells are needed to verify this hypothesis.

In the heart of the Spanish terrapin, formation of cartilage can also be initiated after birth. This is the case for the cartilaginous focus that forms in the wall of the right semilunar valve of the right aorta, entering the fibrous cushion that supports the proximal attachment of the valve leaflet. We were unable to detect any sign of chondrogenesis at these sites in the embryos, nor in the 3-month animal. In the 6-month specimen, however, the focus placed in the aortic sinus wall displayed a relatively advanced stage of development. These data indicate that the formation of the focus had begun between the 3rd and 18th month of life.

The morphogenetic origin of this cartilaginous focus cannot be decided from the present data. Neural crest cells have been found to occur in all aortic and pulmonary valvular cushions of quail-chick chimera embryos (Takamura et al., 1990). Whether such cells also invade the cushions of the cardiac semilunar valves in reptiles remains an open question. The only conclusion derived from our observations at this point in time is that the differentiation into chondrocytes begins much later in the semilunar valve than in the aortico-pulmonary septum and pars fibrosa of the ventricular horizontal septum. However, the reason for this difference is unknown.

The functional significance of the cardiac cartilages remains a problem. It is generally accepted that, when present, they act as pivots resisting mechanical tensions. The deposits form in cardiac portions that are exposed to large stresses during the cardiac cycle, i.e., the cardiac septa and the cardiac valves in reptiles (Matumoto, 1938;
CARDIAC CARTILAGE IN MAUREMYS LEPROSA

The turtle heart begins to beat between the 6th and 7th day of incubation (see Ewert, 1985, for a review of the literature), and the embryonic heart rate varies according to the incubation temperature. In Chelydra serpentina, the estimated mean heart rate amounts to 51.8 beats per minute at 24°C and 72.3 beats per minute at 29°C (Birchard and Reiber, 1996). In the heart of the Spanish terrapin, chondrogenesis can already start between embryonic days 38 and 47, that is, at about 32–41 days after the commencement of the first pulsations. Whether or not the mechanical stimulation generated during this embryonic period on the cardiac structures containing the foci are strong enough to play a primary role in the induction of the chondrogenetic process is an open question.

Another fact that hinders an accurate assessment of the significance of the cardiac cartilage in vertebrates is that its presence is not a prerequisite for normal heart performance. This conclusion relies on the fact that both the incidence and location of the cartilaginous foci vary widely between species and between individuals of the same species (Stiefel, 1926; Matumoto, 1938; Hueper, 1939; Tsusaki et al., 1956; Kashyap, 1959; Wexler, 1964; Hollander, 1968; Kelsall and Visci, 1970; Sans-Coma et al., 1994; López et al., 2001). In the Syrian hamster the location of the cardiac cartilaginous foci is significantly associated with the morphology of the aortic valves; the foci appear where mechanical stress is particularly intense (Sans-Coma et al., 1994). Therefore, it has been suggested that locally strong mechanical stimulation might be a key factor in the formation of cartilage in the vertebrate heart (Matumoto, 1938; Hueper, 1939; Hollander, 1968; Sans-Coma et al., 1994).

Kashyap, 1950; White, 1956,1959; Webb, 1979; Young, 1994; present data) and the attachments of the cardiac semilunar valves to their respective sinuses and the fibrous trigones in both birds (Stiefel, 1926; Matumoto, 1938; Tsusaki et al., 1956; López et al., 2000) and mammals (Matumoto, 1938; Hueper, 1939; Hollander, 1968; Wexler, 1964; Kelsall and Visci, 1970; Sans-Coma et al., 1994; López et al., 2001). In the Syrian hamster the location of the cardiac cartilaginous foci is significantly associated with the morphology of the aortic valves; the foci appear where mechanical stress is particularly intense (Sans-Coma et al., 1994). Therefore, it has been suggested that locally strong mechanical stimulation might be a key factor in the formation of cartilage in the vertebrate heart (Matumoto, 1938; Hueper, 1939; Hollander, 1968; Sans-Coma et al., 1994).

Key factors in the formation of cartilage in the aorticopulmonary septum are the cardiac semilunar valves and the foci appear where mechanical stress is particularly intense (Sans-Coma et al., 1994). Therefore, it has been suggested that locally strong mechanical stimulation might be a key factor in the formation of cartilage in the vertebrate heart (Matumoto, 1938; Hueper, 1939; Hollander, 1968; Sans-Coma et al., 1994).

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notes. However, the reason why some of these cells differentiate into chondrocytes, whereas others do not, remains an open question. It is well known that the morphogenesis of skeletal cartilage is a complex process that involves multiple factors such as the expression of specific genes, synthesis of gene products, and cell interactions (Hall and Newman, 1991; Adolphe, 1992; Hall and Miyake, 1992, 1995; De Crombrugghe et al., 1996; Lefebvre et al., 1998). For the present, however, the role of these factors in the ontogeny of spontaneous extraskeletal cartilages, like those occurring in vertebrate hearts, remains unknown.

So far, the presence of cartilages in the cardiac outflow tract of vertebrates can only be regarded as an outcome of the chondrogenetic potential of this cardiac region (see also Young, 1994; López et al., 2000), yet the expression of this potential presumably relies on the action of various causative factors, which appear to diverge between species, resulting in a wide individual variation.

**LITERATURE CITED**


**Fig. 10.** A: Transverse section of the cardiac outflow tract of a Spanish terrapin age 10 years. Hematoxylin-eosin. Two cartilaginous foci can be seen, one in the aorticopulmonary septum (star) and the other (arrow) in the wall of the right semilunar valve of the right aorta. B: Detail of the area from A (box), showing small, spheroidal chondrocytes embedded in a cellular matrix that stains markedly with hematoxylin. LAo, left aorta; PA, pulmonary artery; RAo, right aorta. Scale bars = 500 μm (A), 100 μm (B).
Lefebvre V, Li P, De Crombrugghe B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 17:5718–5733.


