Biosynthetic Expression of Type X Collagen in Embryonic Chick Sternum Cartilage during Development*

Anthony M. Reginato, James W. Lash, and Sergio A. Jimenez

From the Departments of Medicine and Anatomy of the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

To investigate the temporal and topographic changes in the expression of various collagens during the process of endochondral bone formation, qualitative and quantitative analysis of the collagens synthesized by organ cultures from the separated presumptive calcification regions of embryonic chick sternum at various stages of development was performed. Special emphasis was placed on the study of Type X collagen, a recently described species that may play a role in tissue calcification. We found that Type X collagen is biosynthesized exclusively by cartilage from the zone of presumptive calcification and that its biosynthetic expression is acquired at stage 43 (day 17) of sternal development. Quantitative analysis indicated that Type X was the biosynthetic product which showed the most dramatic changes increasing markedly with increased sternal age. While no Type X collagen could be detected at stage 40, it represented about 12% of the total collagen synthesized at stage 43, further increasing to 45% at stage 46 of sternal development. The increase in Type X collagen in the presumptive calcification region was accompanied by a relative decrease in the proportion of 1, 2α, 3α, 1α(II), and Type IX collagens. In contrast, the permanent hyaline cartilage did not display detectable synthesis of Type X collagen at any sternal age. The strict topographic distribution and the temporal expression of Type X collagen biosynthesis coincident with the development of sternal calcification, confirm the notion that this collagen may play an important role in the extracellular matrix remodeling associated with the initiation and progression of tissue calcification.

Normal development of the skeletal system in vertebrates requires precise temporal and spatial coordination of growth, and reorganization of the various structural elements, particularly cartilage and bone. These changes involve simultaneous *de novo* synthesis and deposition of matrix and removal of the pre-existing matrix. During long bone development, the matrix which is initially comprised of hyaline cartilage undergoes appositional growth to form a dumbbell-shaped mass surrounded by perichondrium with a central portion destined to become the bone diaphysis and the two distal portions, or epiphyses, which will form the future articular regions. The cartilage matrix participates in endochondral bone formation by becoming calcified. During this process, the chondrocytes become hypertrophic and subsequently degenerate leaving the interconnecting spaces for osteogenesis (1). In addition to endochondral ossification, intramembranous ossification is involved in the skeletal development of mammals and birds. This process involves bone formation within fibrocartilage membranes of condensed primitive mesenchymal tissue. Mesenchymal cells differentiate into osteoblasts which begin synthesis and secretion of osteoid matrix at the centers of ossification. The deposited osteoid rapidly becomes mineralized.

It has been well documented that the hyaline cartilage matrix involved in endochondral bone formation exhibits a high degree of molecular heterogeneity particularly regarding its collagenous components (2). Although it is generally accepted that skeletal development involves primarily changes in Type II the principal cartilage collagen, and Type I the main bone collagen (3–5), the role of the more recently identified minor cartilage collagens designated as 1α, 2α, 3α, 6, 7, Type IX (8–13), and Type X (14–22) has not been fully examined.

In the present paper, we have studied the expression of the various collagens in embryonic chick cartilage during the developmental process leading to its calcification. We found that organ cultures of embryonic chick sternal cartilage exhibit profound spatial and temporal changes in the expression of Type X collagen biosynthesis during differentiation. The results demonstrated that Type X collagen biosynthesis is exclusively confined to cartilage from the presumptive calcification region and that its expression is acquired only at stage 43 of sternal development. A marked acceleration of Type X biosynthesis occurred at stage 46, corresponding with the onset of ossification (23, 24).

**EXPERIMENTAL PROCEDURES**

**Biosynthetic Studies**—Sterna from White Leghorn chick embryos corresponding to developmental stages 40 (14 day), 43 (17 day), and 46 (20 day) as described by Hamburger and Hamilton (31) were removed and carefully dissected from surrounding perichondrium. The caudal two-thirds comprising the permanent hyaline and the cephalic one-third comprising the presumptive calcification cartilage, respectively, were separated with a scalpel. Microscopic examination confirmed that the cephalic portion obtained with this procedure contained almost entirely hypertrophic chondrocytes. The tissues were incubated in Eagle’s minimal essential medium containing ascorbic acid (100 μg/ml), β-aminopropionitrile (2.5 mM), and 1% streptomycin. Cultures were labeled for 48 h with 1 μCi/ml [3H] proline (275 mCi/mmol, Amersham Corp.). After incubation, the media were removed and frozen after a solution of protease inhibitors was added to give a final concentration of 50 mM disodium EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5.0 mM N-ethylmaleimide, and 1.0 mM p-amino benzamidine/HC1.

**Collagen Solubilization**—After labeling, the minced cartilage was...
homogenized with a Polytron homogenizer for 3 min in a buffer containing 1.0 M NaCl, 50 mM Tris-HCl, pH 7.4, at 4°C and the concentrations of protease inhibitors listed above. The homogenized tissues were extracted with the same buffer for 72 h at 4°C. The solubilized material was removed by centrifugation and insoluble material was sequentially extracted for a number of times in a manner previously described by Jimenez and Bashey (25). Briefly, this involved sequential extraction with first 0.5 M acetic acid, then with 0.5 M acetic acid containing 1 mg/ml pepsin (Sigma), followed by two extractions with a solution containing 0.15 M NaCl, 50 mM Tris-HCl, 25 mM dithiothreitol (DTT), pH 7.4, at 4°C and finally a repeated extraction with the 0.5 M acetic acid/1 mg/ml pepsin solution. This method resulted in complete solubilization of the tissues. Following centrifugation, the supernatants were removed and after extensive dialysis against 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, buffer, aliquots from each of the extracts were taken for determination of their [14C]hydroxyproline content by the method of Juva and Frockop (26).

Proteolytic Digestion—Lyophilized samples used for collagenase digestion were resuspended in a buffer containing 0.15 M NaCl, 5.0 mM CaCl2, 50 mM Tris-HCl, 10 mM N-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.4, at 37°C. Purified bacterial collagenase (Form III, Advance Biofactures) was added (150 units/ml) and the samples were digested for 6 h at 37°C. The reaction was terminated by addition of EDTA to a final concentration of 10 mM and cooling at 4°C. For digestion with pepsin, lyophilized samples were suspended in 0.5 M acetic acid containing 300 mg/ml pepsin and incubated for 6 h at 15°C. The reaction was terminated by addition of 0.1 ml of 6 M NaOH and the digested samples were then dialyzed at 4°C against 0.1 M ammonium bicarbonate and lyophilized.

Protein and Collagen Biosynthesis by Permanent Hyaline and Presumptive Calcification Cartilage at Three Stages of Development—Determination of total [14C]proline incorporation and [14C]hydroxyproline synthesis by cartilage from the separate sternal regions at the three stages of development showed that the presumptive calcification region exhibited 2-fold greater [14C]proline incorporation and about 3-fold greater [14C]hydroxyproline synthesis at the three sternal ages. Similarly, the degree of proline hydroxylation in the newly synthesized proteins was higher in the presumptive calcification cartilage at three stages of development (44.5, 46.7, and 45.7% versus 36.9, 40.3, and 40.6%, respectively).

Electrophoretic and fluorographic examination of the 1.0 M NaCl extracts from the two separate regions at stage 40 of sternal development before and after proteolytic digestion is shown in Fig. 1. The pattern of labeled proteins present in the samples from both regions under reducing conditions (lanes 2 and 6) shows a prominent band corresponding to...
Type X Collagen Biosynthesis in Chick Embryo Development

Extractability of newly synthesized collagen from the cartilaginous region and presumptive calcification regions of stages 40, 43, and 46 embryonic chick sternum

Embryonic chick sterna from stages 40, 43, and 46 were separated into permanent hyaline (caudal) and presumptive (cephalic) cartilaginous regions, minced, and labeled with [14C]proline as described under "Experimental Procedures." After labeling the tissue was homogenized in 1.0 M NaCl, 50 mM Tris-HCl, pH 7.4, buffer and sequentially extracted as described previously (25). Aliquots of the extracts were hydrolyzed after extensive dialysis and their [14C]hydroxyproline content assayed by the method of Juva and Prockop (26).

<table>
<thead>
<tr>
<th>Extractants</th>
<th>Permanent hyaline cartilage</th>
<th>Presumptive calcification cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryonic stages</td>
<td>[14C]Hydroxyproline</td>
</tr>
<tr>
<td>1.0 M NaCl + 0.5 M acetic acid</td>
<td>40</td>
<td>617.8</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>1624.3</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>1530.6</td>
</tr>
<tr>
<td>Pepsin (1 mg/ml)</td>
<td>40</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>28.9</td>
</tr>
<tr>
<td>DTT + pepsin after DTT</td>
<td>40</td>
<td>113.7</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>276.3</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>275.9</td>
</tr>
</tbody>
</table>

The labeled molecules present in the 1.0 M NaCl extracts from stage 43 showed marked differences between the two regions (Fig. 2A, lane 1 and Fig. 2B, lane 1). In the extracts from the presumptive calcification region a prominent band migrating with an apparent Mr = 60,000 was observed and represented about 16% of the total radioactivity migrating into the gel. This band was absent in the region of permanent hyaline cartilage. The Mr, 60,000 band did not change its electrophoretic mobility under reducing and nonreducing conditions (Fig. 2B, lanes 1 and 2). After pepsin digestion the Mr = 60,000 band migrated into a 45,000 Mr, position indicating proteolytic removal of noncollagenous domains (Fig. 2B, lane 3). The migration of the 45,000 Mr was unchanged under reducing and nonreducing conditions (Fig. 2B, lane 4). The electrophoretic mobility of this band is consistent with the behavior of Type X collagen before and after pepsin treatment (14, 15, 18, 28). The collagenous nature of the labeled proteins migrating in this position was confirmed by their disappearance after collagenase digestion (not shown). Similar analysis of the permanent hyaline cartilage region (Fig. 2A, lanes 1–4) failed to show any radioactive bands corresponding to Type X collagen. Additional bands migrating below α1(II) chains in the pepsin-digested samples from both regions displayed electrophoretic mobilities similar to those of Type IX collagen after pepsin digestion and disulfide bond reduction as previously described (8, 13, 19).

The labeled molecules present in the 1.0 M NaCl extracts from stage 46 sterna showed a similar pattern to those from sterna at stage 43 of development (Fig. 3B). The Mr, 60,000 band corresponding to Type X collagen was found exclusively in the presumptive calcification region and represented approximately 38% of the total radioactivity migrating into the gel (Fig. 3A, lane 1 and Fig. 3B, lane 1). Pepsin digestion also resulted in the removal of noncollagenous proteins yielding the expected pepsin-cleaved Type X (Mr = 45,000) band (Fig. 3B, Lanes 3 and 4). The migration pattern under reducing and nonreducing conditions did not change for either form of Type X collagen (Fig. 3B, lanes 3–4). The dramatic increase in the proportion of Type X collagen in the pepsin-digested extracts from the presumptive calcification region during development from stage 40 to 46 was accompanied by a concomitant decrease in the proportion of Type IX collagen.

α1(II) and other minor bands migrating above it. Three of these bands correspond to the pre-pepsin forms of 1α, 2α, and 3α and the remaining bands appear to correspond to intact and partially processed Type II procollagen molecules. As expected, after pepsin digestion these molecules migrated more rapidly due to removal of their noncollagenous extensions. As described previously, 1α and 2α, migrated above α1(II) and 3α co-migrated with it. The electrophoretic patterns obtained under reducing and nonreducing conditions from intact and pepsin-treated extracts from both regions were essentially identical (Fig. 1, lanes 1–4 and 5–8). Bands with electrophoretic migration corresponding to Type IX and Type X collagens were not detected in either region at stage 40 of development even when overloaded samples were examined.

The electrophoretic analysis of the labeled collagens present in the 1.0 M NaCl extracts at stage 43 showed marked differences between the two regions (Fig. 2A, lane 1 and Fig. 2B, lane 1). In the extracts from the presumptive calcification region a prominent band migrating with an apparent Mr = 60,000 was observed and represented about 16% of the total radioactivity migrating into the gel. This band was absent in the region of permanent hyaline cartilage. The Mr, 60,000 band did not change its electrophoretic mobility under reducing and nonreducing conditions (Fig. 2B, lanes 1 and 2). After pepsin digestion the Mr = 60,000 band migrated into a 45,000 Mr, position indicating proteolytic removal of noncollagenous domains (Fig. 2B, lane 3). The migration of the 45,000 Mr was unchanged under reducing and nonreducing conditions (Fig. 2B, lane 4). The electrophoretic mobility of this band is consistent with the behavior of Type X collagen before and after pepsin treatment (14, 15, 18, 28). The collagenous nature of the labeled proteins migrating in this position was confirmed by their disappearance after collagenase digestion (not shown). Similar analysis of the permanent hyaline cartilage region (Fig. 2A, lanes 1–4) failed to show any radioactive bands corresponding to Type X collagen. Additional bands migrating below α1(II) chains in the pepsin-digested samples from both regions displayed electrophoretic mobilities similar to those of Type IX collagen after pepsin digestion and disulfide bond reduction as previously described (8, 13, 19).

The labeled molecules present in the 1.0 M NaCl extracts from stage 46 sterna showed a similar pattern to those from sterna at stage 43 of development (Fig. 3B). The Mr, 60,000 band corresponding to Type X collagen was found exclusively in the presumptive calcification region and represented approximately 38% of the total radioactivity migrating into the gel (Fig. 3A, lane 1 and Fig. 3B, lane 1). Pepsin digestion also resulted in the removal of noncollagenous proteins yielding the expected pepsin-cleaved Type X (Mr = 45,000) band (Fig. 3B, Lanes 3 and 4). The migration pattern under reducing and nonreducing conditions did not change for either form of Type X collagen (Fig. 3B, lanes 3–4). The dramatic increase in the proportion of Type X collagen in the pepsin-digested extracts from the presumptive calcification region during development from stage 40 to 46 was accompanied by a concomitant decrease in the proportion of Type IX collagen.

Fig. 1. SDS-polyacrylamide gel electrophoresis fluorograph of 1.0 M NaCl extracts from permanent hyaline cartilage and presumptive calcification cartilage from stage 40 sternum. Samples of intact or pepsin-digested 1.0 M NaCl extracts were denatured and electrophoresed under reducing and nonreducing conditions in the presence or absence of 1% (v/v) β-mercaptoethanol and 1% SDS as described under "Experimental Procedures" and were electrophoresed in 4–10% polyacrylamide exponential gels as described previously (21, 22). Lane 1, permanent hyaline cartilage extract without reduction before pepsin digestion; lane 2, as lane 1 after reduction; lane 3, pepsin-digested extract without reduction; lane 4, as lane 3 after reduction; lane 5, presumptive calcification cartilage extract without reduction before pepsin digestion; lane 6, as lane 5 after reduction; lane 7, pepsin-digested extract without reduction; lane 8, as lane 7 after reduction.
polypeptides from 19 to 5% and a reduction in the proportion of \( \alpha_1 \) and \( \alpha_2 \) collagen from 22 to 11%.

Electrophoretic analysis of the DTT extracts is shown in Fig. 4. The electrophorograms of these extracts disclosed dramatic qualitative and quantitative changes in the expression of Type X collagen in cartilage from the presumptive calcification cartilage with increasing developmental age. Whereas no radioactivity migrating in the position of pepsin-cleaved Type X collagen \( (M_r = 45,000) \) could be detected in samples from stage 40, even when overloaded gels were examined, large amounts of radioactivity were found migrating in this region at stages 43 and 46 of development. Type X comprised about 11% of the total radioactivity at stage 43 sternum. This proportion increased to 45% at stage 46 of development. Bands corresponding to Type X collagen were not detected at all in the extracts from the permanent hyaline cartilage at any of the three stages. The figure also shows that the extracts from both zones at the three developmental stages exhibited several bands migrating below \( \alpha_1(II) \) chains. The apparent molecular weight of these bands estimated by comparison to the migration of Type II collagen CNBr peptides was calculated to be in decreasing order: 80,000, 69,000, 54,000, and 49,000 \( M_r \). In most experiments the 69,000 \( M_r \) band was the most prominent. These bands are similar to those observed after pepsin digestion and subsequent reduction of Type IX collagen polypeptides described by Gibson et al. (19) and Yasui et al. (13).

**CNBr Peptide Pattern Analysis**—In order to further characterize the collagens present in the pepsin-digested 1.0 M NaCl extracts of the presumptive calcification region, two-dimensional CNBr mapping of extracts from both regions of stage 46 sterna was performed. Comparison of the peptide patterns obtained from the permanent hyaline and presumptive calcification cartilage extracts showed the presence of the major CNBr peptides of Type II collagen. The four prominent peptides observed (arrowheads, Fig. 5) correspond in decreasing order to CB 10.5 (31,000 \( M_r \)), CB 11 (25,000 \( M_r \)), CB 8 (13,000 \( M_r \)), and CB 9.7 (10,800 \( M_r \)). The major CNBr peptides of Type X collagen exhibited a \( M_r \) below 13,000 and were found only in the presumptive calcification region (small
Type X Collagen Biosynthesis in Chick Embryo Development

Type X collagen biosynthesis was characterized in chick embryo development. The biosynthesis of Type X collagen was studied by examining the incorporation of labeled proline into collagen chains. Two-dimensional CNBr peptide mapping was performed to analyze the temporal and spatial expression of collagenous molecules during development.

Quantitative Analysis of the Relative Proportion of Collagen Synthesized by the Developing Embryonic Sterna—To compare the relative proportion of the various collagen syntheses by the separate sternal regions during development, fluorographs of the 1.0 M NaCl and DTT extracts were examined by densitometric analysis and the areas under each collagen chain were calculated by planimetry. The results shown in Table II indicate that the sternum exhibits both spatial and temporal expression of collagenous molecules during embryonic development. The permanent hyaline cartilage region displayed a constant relative proportion of 3α1 + α1(II) collagen throughout development, whereas the proportion of 1α1 + 2α1 decreased from 29.5% at stage 40 to 21% at stage 43 and 23% at stage 46. On the other hand, the proportion of Type IX collagen exhibited an increase from 9% at stage 40, to 18% at stage 43, and to 19% at stage 46 of development. As expected, the permanent hyaline cartilage region did not display any detectable biosynthesis of Type X collagen.

Type X collagen comprised approximately 12% of the newly synthesized collagen from the presumptive calcification region at stage 43 of development. The increase in Type X collagen was accompanied by a decrease in the relative proportion of all other collagens. Type IX collagen declined from 11.9% at stage 40 to 5.2% at stage 46; while the 1α1 and 2α1 chains declined from 32% to 13% and 3α1 + α1(II) from 56 to 37%. A comparison of the rates of protein synthesis and collagen biosynthesis corrected for the absolute number of cells obtained from each of the separate regions demonstrated that chondrocytes from the presumptive calcification region were substantially more active than those from the zone of permanent hyaline cartilage at both sternal stages (Table III). At stage 40, the presumptive calcification chondrocytes incorporated two times as much[^14C]proline and synthesized 2.5 greater amounts of[^14C]hydroxyproline when calculated on a per cell basis. Similarly, at stage 46 of development, cells from the presumptive calcification region incorporated 2.2 times as much[^14C]proline and synthesized about 3 times more[^14C]hydroxyproline than cells from the permanent hyaline cartilage region. When the rates of synthesis of Type II and Type X collagens synthesized per cell by the permanent hyaline and presumptive calcification chondrocytes were examined, it was found that Type X collagen represented the major biosynthetic product of presumptive calcification chondrocytes at stage 46, since these cells synthesized essentially equal amounts of Type X and Type II collagens. Furthermore, a comparison of the amount of Type X collagen produced by stage 46 presumptive calcification chondrocytes to the amount of Type II collagen produced by permanent hyaline cartilage chondrocytes at the same stage demonstrated that the presumptive calcification cartilage chondrocytes synthesized 250% more Type X collagen than the amount of Type II collagen synthesized by the permanent hyaline cartilage chondrocytes.

Morphologic Changes during the Development of Embryonic Sterna—To correlate the changes in collagen biosynthetic expression with gross morphologic changes occurring in the developing chick sternum, whole sterna from embryos at stages 40, 43, and 46 of development were differentially stained for proteoglycans with alcian blue (Fig. 6A) and for calcified arrows). These results are similar to the CNBr peptide pattern of Type X collagen from chick embryo tibiotarsus (14, 15) or from long term culture of chick chondrocytes grown in three-dimensional gels (18).
The relative proportion of various collagen chains synthesized by the permanent hyaline and presumptive calcification regions of embryonic chick sternum at various stages of development.

The relative proportion of various collagen chains was calculated from electrophoretic analysis of pepsin-digested 1.6 M NaCl extracts and DTT extracts from the permanent hyaline cartilage and presumptive calcification regions as described under "Experimental Procedures." After fluorography the gels were scanned at 540 nm in a linear drive densitometer and the areas under the peaks corresponding to each of the collagen chains were calculated with a planimeter. The values shown are the averages of values obtained with two separate preparations of extracts.

<table>
<thead>
<tr>
<th>Sternal region</th>
<th>Embryonic stage</th>
<th>Proportion of each collagen chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$1 \alpha + 2 \beta$</td>
</tr>
<tr>
<td>Permanent hyaline cartilage</td>
<td>Stage 40</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>Stage 46</td>
<td>20.7</td>
</tr>
<tr>
<td>Permanent hyaline cartilage</td>
<td></td>
<td>23.1</td>
</tr>
<tr>
<td>Presumptive calcification region</td>
<td>Stage 40</td>
<td>32.7</td>
</tr>
<tr>
<td>Presumptive calcification region</td>
<td>Stage 46</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

A significant finding of this study is the demonstration of a spatial and temporal expression of Type X collagen biosynthesis by the presumptive calcification region in the developing embryonic chick sternum. The Type X collagen synthesized by the embryonic chick sternum organ cultures was characterized by electrophoretic analysis, proteolytic digestion, and CNBr-peptide mapping and it was shown to be similar to Type X collagen from chick embryos (14, 15) and from cultures of chick chondrocytes grown in three-dimensional gels (18, 20). In agreement with these studies we found that tissue extracts obtained without protease digestion contained intact Type X collagen which migrate electrophoretically with an apparent $M_r$ of 60,000. These chains do not contain disulfide bonds since they do not change electrophoretic mobility after reduction. Limited proteolytic digestion of the extracts results in cleavage of non-helical regions of these chains yielding 45,000 $M_r$ probes.

Comparison of the rates of $[^{14}C]$proline incorporation, $[^{14}C]$hydroxyproline synthesis, and production of the various collagens on a cell basis between permanent hyaline and presumptive calcification regions of embryonic sternum at stages 40 and 46 of development.

**Table II**

<table>
<thead>
<tr>
<th>Collagen Collagen</th>
<th>Permanent hyaline</th>
<th>Presumptive calcification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 40</td>
<td>Stage 46</td>
</tr>
<tr>
<td></td>
<td>$dpm \times 10^{10}$</td>
<td>$dpm \times 10^{10}$</td>
</tr>
<tr>
<td>Permanent hyaline</td>
<td>78.9</td>
<td>81.0</td>
</tr>
<tr>
<td>cartilage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total $[^{14}C]$</td>
<td>216.7</td>
<td>199.5</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>445.5</td>
<td>523.1</td>
</tr>
<tr>
<td>Total $[^{14}C]$Hydroxyproline in&quot;</td>
<td>28.4</td>
<td>42.2</td>
</tr>
<tr>
<td>$1 \alpha + 2 \beta$ collagen</td>
<td>110.6</td>
<td>94.1</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>3.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Type IX collagen</td>
<td>0</td>
<td>98.7</td>
</tr>
<tr>
<td>Type X collagen</td>
<td>0</td>
<td>98.7</td>
</tr>
</tbody>
</table>

The amount of $[^{14}C]$hydroxyproline in each collagen was calculated from densitometric analysis of fluorographs from the pepsin-treated neutral salt and the DTT extracts with two separate preparations of extracts and were corrected by the number of cells obtained after enzymatic dissociation of sternum as described under "Experimental Procedures." Each value represents an average of two separate experiments and is expressed as $dpm \times 10^{10}$ cells/48 h.

- The amount of $[^{14}C]$hydroxyproline synthesized in each collagen was calculated from densitometric analysis of fluorographs from the pepsin-treated neutral salt and the DTT extracts with two separate preparations of extracts and were corrected by the number of cells obtained after enzymatic dissociation of chondrocytes in parallel experiments.

- In selected cases direct determination of $[^{14}C]$hydroxyproline synthesis between the two regions showed that the presumptive calcification region in the developing embryonic chick sternum was substantially more active than the permanent hyaline region at the three stages analyzed since it displayed 2-fold greater $[^{14}C]$proline incorporation and about 2.5-3-fold greater $[^{14}C]$hydroxyproline biosynthesis.

**Table III**

Comparison of the rates of $[^{14}C]$proline incorporation, $[^{14}C]$hydroxyproline synthesis, and production of the various collagens on a cell basis between permanent hyaline and presumptive calcification regions of embryonic sternum at stages 40 and 46 of development.

**Discussion**

A significant finding of this study is the demonstration of a spatial and temporal expression of Type X collagen biosynthesis by the presumptive calcification region in the developing embryonic chick sternum. The Type X collagen synthesized by the embryonic chick sternum organ cultures was characterized by electrophoretic analysis, proteolytic digestion, and CNBr-peptide mapping and it was shown to be similar to Type X collagen from chick embryos (14, 15) and from cultures of chick chondrocytes grown in three-dimensional gels (18, 20). In agreement with these studies we found that tissue extracts obtained without protease digestion contained intact Type X collagen which migrate electrophoretically with an apparent $M_r$ of 60,000. These chains do not contain disulfide bonds since they do not change electrophoretic mobility after reduction. Limited proteolytic digestion of the extracts results in cleavage of non-helical regions of these chains yielding 45,000 $M_r$ probes.

Comparison of the rates of $[^{14}C]$proline incorporation, $[^{14}C]$hydroxyproline synthesis, and production of the various collagens on a cell basis between permanent hyaline and presumptive calcification regions of embryonic sternum at stages 40 and 46 of development.
collagen and increased to 45% at stage 46 of sternal development. The increase in Type X was accompanied by a decrease in the relative proportion of $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_1$II, and Type IX collagens. In contrast, the permanent hyaline cartilage exhibited an increase in the proportion of Type IX collagen and a slight decrease in the proportion of $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_1$II. The mechanisms responsible for the striking changes in the relative proportions of the various collagen subpopulations with sternal development observed in the presumptive calcification region are not clear. The decrease in levels of $\alpha_1$, $\alpha_2$, and $\alpha_3$ collagens, which are referred to as the cell-associated collagens (31, 32), may be related to the loss of membrane integrity and release of lysosomal content from chondrocytes undergoing calcification. The decrease of Type IX collagen in the presumptive calcification region with sternal age may represent the transformation of the permanent hyaline cartilage matrix to one of calcification. The dramatic increase in Type X collagen production may also reflect changes occurring on the extracellular matrix upon the onset of calcification.

The results of quantitative analysis at stage 46 of development demonstrated that Type X collagen represents a major biosynthetic product of these cells and when calculated on a per cell basis it was found that a chondrocyte from the presumptive calcification region produced 250% greater amounts of Type X collagen than the amount of Type II collagen produced by a chondrocyte from the permanent hyaline cartilage region.

Whether the initiation of Type X collagen biosynthesis at stage 43 of development demonstrated here reflects changes in chondrocyte gene expression or is the result of expansion of a unique subpopulation of chondrocytes selectively committed to Type X collagen production, is not known. Nevertheless, the strict topographical and temporal distribution of Type X collagen synthesis coincident with initiation of sternal calcification indicates that cells within the presumptive calcification region are intimately involved in the process of endochondral bone formation.

In recent studies, Capasso et al. (33, 34) and Schmid and Linsenmayer (17) showed biosynthetic and immunohistochemical acquisition of Type X collagen in the developing tibiotarsus. Capasso et al. (34) suggested that the Type X production may be the sequela of blood vessel ingrowth, in which a serum factor might be responsible for initiating its synthesis. The studies reported here, however, indicate that the earliest biochemically detectable synthesis of Type X collagen occurred at stage 43, before the appearance of any form of vascularization in the tissue, which is usually observed at the later stage of development (stage 46). It should be noted, however, that maximal Type X collagen biosynthesis was coincident with morphological observations of proteoglycan depletion and Ca$^{2+}$ deposition at the most cephalad region of the presumptive calcification cartilage. Thus, it could be postulated that Type X collagen could provide a permissive matrix for calcification, but the process of calcification being under a different set of regulatory controls. Elucidation of this possibility, however, will require in vitro studies on the calcification of Type X collagen and the demonstration of mineral deposits characteristic of calcification after tissue implantation of purified Type X collagen. These studies are currently being performed.

Acknowledgments—The expert assistance of E. Lobb in preparation of the manuscript is gratefully acknowledged.

REFERENCES