TYPE IX AND TYPE II COLLAGENS ARE COORDINATELY EXPRESSED DURING CHICK LIMB DEVELOPMENT

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ABSTRACT

Changes in the level of type IX and type II collagen mRNA were examined during the course of chick limb development *in vivo* using cloned cDNA probes. Substantial amounts of type IX and type II collagen mRNA were detected at stage 26, concurrently with the chondrogenic differentiation of limb mesenchyme. Subsequently, the amounts of type IX and type II collagen mRNA showed parallel and marked increases corresponding to the progressive accumulation of cartilage matrix in the limb. Type IX and type II collagen synthesis also increased coinciding with a dramatic increase in their respective mRNAs. The present study suggests that type IX and type II collagens are coordinately expressed during the course of limb cartilage differentiation, which would enable the formation of a very precise temporal and spatial arrangement of cartilage matrices.

Differentiation of cartilage is an important key event in vertebrate limb development and has been described extensively (4, 8, 21, 22, 24). Loosely packed mesenchyme cells of chick limb buds start to condensate in the limb core at stage 22 (6). By stage 25/26, these cells begin to secrete matrix macromolecules making a mold which is subsequently replaced by bone. Biochemically, this process of cartilage differentiation is characterized by the initiation of type II, type IX, and type XI collagen synthesis and cessation of type I collagen synthesis (9, 14, 27). The molecular regulation of these events is still obscure, but to analyze it will be of vital importance for understanding the mechanism of cartilage differentiation.

Recently, the availability of cloned cDNA

probes complementary to mRNA for collagens and cartilage proteoglycan has made it possible to analyze their gene expression during limb cartilage differentiation (10-12, 15). In the work presented here, we have used cDNA probes coding for type IX and type II collagens to examine the mRNA levels in chick limbs undergoing cartilage differentiation. Changes in collagen synthesis were also examined. Our results suggest that type IX and type II collagens are coordinately expressed during the process of cartilage differentiation. This is quite reasonable since type IX and II collagens are covalently cross-linked (3, 25) and would participate coordinately in the formation of cartilage structure.

MATERIALS AND METHODS

RNA Extraction

Forelimbs (wing of wing bud) were excised

Abbreviations: 1×SSC, 0.15 M NaCl in 0.015 M sodium citrate buffer, pH 7.0; SDS, sodium dodecyl sulfate

from chick embryos at stage 22 to 32 (6). After homogenization with a Polytron homogenizer, the total RNA was prepared by guanidine thiocyanate extraction/CsCl density gradient centrifugation procedure (2).

Dot Blot and Northern Blot Hybridizations

Dot blots of total RNA were performed as described (5). For Northern blot analysis, the total cellular RNA (7 to 10 μ g) from different stage forelimbs was electrophoresed in 0.8% agarose gels in the presence of formaldehyde/formaldehyde and transferred onto nitrocellulose filters. The filters were hybridized to nick-translated DNA as described previously (23). After hybridization in $5\times$ SSC (0.15 M NaCl in 0.015 M sodium citrate buffer, pH 7.0) and 1% *N*-lauroylsarcosine at 65°C, the filters were washed twice in $3\times$ SSC and 0.5% *N*-lauroylsarcosine, twice in $3\times$ SSC at 65°C, and exposed to Kodak Royal X-Omat film at -70°C. The levels of hybridizable RNA sequences were quantified by scanning the dot blot autoradiograms with a scanning densitometer (CS-910 scanner, Shimadzu).

The collagen cDNA probes used as hybridization probes were pYN1738, a 3294 base-pair cDNA encoding part of the $\alpha 1(\text{IX})$ collagen subunit, pYN2142, a 603 base-pair cDNA encoding part of the $\alpha 1(\text{II})$ procollagen subunit, and pYN 535, a 530 base-pair cDNA encoding part of the $\alpha 2(\text{I})$ procollagen subunit (17, 18) (kindly provided by Drs Y. Ninomiya and B. R. Olsen, Harvard Medical School).

Analysis of Collagen Synthesis

Collagen newly synthesized by the chick forelimbs was analyzed as previously reported (9). The excised forelimbs from stage 24 to 32 chick embryos were labeled *in vitro* for 6 h at 37°C with [^{14}C]proline (20 $\mu\text{Ci/ml}$, New England Nuclear) and the synthesized collagens were extracted and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography (1, 13). To quantify the collagen phenotype, the fluorographic bands were traced on the dried gels, cut out into scintillation vials, and hydrolyzed in 0.5 ml of 0.4 M NaOH at 56°C for 24 h. After neutralization with HCl, the radioactivity of the individual bands was counted in 10 ml of PCS II (Amer-

sham).

RESULTS AND DISCUSSION

The cDNA fragments used as hybridization probes are shown in Fig. 1. The restriction fragments of pYN 535 and pYN 2142 were selected so as to show the least possible cross-hybridization as described previously (7). The whole insert was used for pYN 1738 because it did not show any cross-hybridization under the present hybridization conditions (see below).

Type IX and Type II Collagen mRNA Levels during Limb Development

To examine how the levels of type IX collagen and type II collagen RNA change during limb development, total cellular RNAs isolated from chick forelimbs at stages 24, 26, 28, 30, 32 of development were probed with type IX- and type II-collagen-specific [^{32}P]-labeled cDNAs. This procedure allowed us to quantify relative changes in total hybridizable type IX and type II collagen mRNAs.

No detectable amounts of mRNA hybridized to type IX collagen cDNA was present at stage 24 (Fig. 2), which is a stage prior to the occurrence of morphological indications of chondrogenic differentiation by the mesenchyme cells. By stage 26, which corresponds

Fig. 1 Diagram showing the location of collagen cDNA probes. Regions used as hybridization probes are indicated by wide black bars. The direction of the coding strand of the probe is from left to right, as indicated by the 5' and 3' notation. The 3'-untranslated portion is indicated by a dashed line. Three collagenous domains (COL 1, COL 2 and COL 3) of type IX collagen are indicated according to reference 17.

Fig. 2 Dot hybridization analysis of $\alpha 1(\text{IX})$ collagen and $\alpha 1(\text{II})$ procollagen mRNA levels in the developing chick limbs. Two μg of total RNA extracted at the stage indicated were blotted on nitrocellulose and hybridized to the indicated nick-translated probes.

to the onset of overt chondrogenesis (4, 21), substantial amounts of type IX collagen mRNA were detected. This initiation of type IX collagen mRNA accumulation coincided with a dramatic increase in type II collagen mRNA accumulation (Fig. 2). The amounts of type IX and type II collagen mRNA began to increase dramatically after this stage which paralleled the progressive accumulation of cartilage matrix in the limb core. Judging from the results of densitometric scanning, the level of mRNA at stage 32 is approximately 12 times higher for type IX collagen and 7 times higher for type II collagen than at stage 26. It should be noted, however, that a very low level of type II collagen mRNA was detected at stage 24, prior to the onset of chondrogenesis as reported previously (11, 12). This low level of mRNA was not detectable in the following Northern blot analysis because of the lower sensitivity of the Northern blot hybridization method.

To confirm that the observed increase in type IX and type II collagen mRNA levels was not an effect of cross-hybridization to other collagenous mRNA species or an artifact of the RNA preparation, Northern blot hybridization was performed. Although the relative amounts of each mRNA species from different stages are not precisely shown in Fig. 3, it was obvious that the $\alpha 1(\text{IX})$ collagen probe was capable of hybridizing to two RNA bands of approximately 4.2 kilobases and 3.5 kilobases, and the $\alpha 1(\text{II})$ collagen probe to one RNA band of 5.9 kilobases. The presence of

Fig. 3 Northern blot hybridization of total RNA. Approximately 7 to 10 μg of total RNA were electrophoresed in a 0.8% agarose gel, blotted onto nitrocellulose, and hybridized to the indicated probes. Chicken sternal cartilage 18S and 27S rRNAs were used as RNA size markers.

two $\alpha 1(\text{IX})$ mRNAs of different size is probably due to differences in the utilization of multiple polyadenylation sites as previously documented (17). There was no cross-hybridization to other collagen mRNA species. The finding that the relative mRNA level of the $\alpha 2(\text{I})$ chain remained approximately constant is not surprising since limbs at stages between 24 and 32 predominantly consist of non-cartilaginous tissue which synthesizes type I collagen. In addition, $\alpha 2(\text{I})$ mRNA is present even in cartilage where no $\alpha 2(\text{I})$ collagen chain synthesis is detectable (20).

Type IX and Type II Collagen Synthesis

Newly synthesized collagens were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the changes in collagen phenotype are summarized in Fig. 4. Utilizing the known chain composition of collagen type I, II, and IX, the relative amount of each collagen was calculated from the radioactivity of electrophoretic bands (28). The colla-

Fig. 4 Change in collagen phenotype during chick limb development. Collagen from stage 24–32 forelimbs was fractionated by electrophoresis, and the radioactive bands were removed from the gels and counted as described under Materials and Methods. The amount of each collagen type was calculated according to reference 28. Because of the method of calculation, a small proportion of type I trimer is included in the type II collagen. Values are expressed as the mean from duplicate experiments. *Inset:* The amount of radioactive type IX and type II collagens at each point is expressed as an amount relative to that present at stage 32, which was arbitrarily set to 100.

gen synthesized by stage 24 limb buds was predominantly type I, while neither type IX nor type II collagen was detected. Type IX and type II collagen synthesis was first detected at stage 26, when phenotypically identifiable cartilage is first detected in the limb. Subsequently, the synthesis of type IX collagen increased dramatically, parallel to an increase in type II collagen synthesis. This dramatic and parallel increase in type IX and type II collagen synthesis is further obvious when the amount of radioactive collagens at each point is expressed as an amount relative to that present at stage 32 (Fig. 4, *inset*). These results suggest that the expression of type IX and type II collagens is coordinately regulated during the development of the chick limb. Since the dramatic increase in the collagen synthesis paralleled the increase in the respective mRNA levels, their regulation must, at least in part, be pretranslational.

Further support for the assumption that the expression of type IX and type II collagens is coordinately regulated can be derived from our previous observation (28). We analyzed

collagen synthesis during the growth of chick chondrocytes in retinoic acid or 5-bromo-2'-deoxyuridine. The synthesis of type IX and type II collagens showed parallel decrease after treatment with these two different modulating agents. Thus, these collagens appear to form a group of coordinately regulated molecules associated with the differentiated chondrogenic phenotype.

The function of type IX collagen is still obscure. However, its localization within the cartilage is suggestive of a structural role. It has been demonstrated that type IX collagen is closely associated with the fibrils formed by type II collagen molecules (16). In addition, van der Rest and Mayne (25), and Eyre *et al.* (3) have demonstrated the existence of a covalent cross-link between type IX collagen and type II collagen. This finding led van der Rest and Mayne to the hypothesis that type IX collagen interacts with the type II fibril, with lateral association between the type II triple-helices and C-terminal 2/3 of type IX collagen molecules (25). The opposite end of the type IX collagen molecule, the N-terminal globular domain, is basic ($pI=9.7$) (26) and may serve as a junction site with other matrix components, such as cartilage glycosaminoglycan (19). Thus, type IX collagen is thought to play a role in the interaction between cartilage matrix components.

As demonstrated in the present study, the expression of type IX and type II collagens is coordinately regulated, as shown by parallel changes in mRNA and protein synthesis during the course of limb development. Furthermore, Kosher *et al.* (10) have demonstrated that the cartilage proteoglycan core protein and type II collagen genes are coordinately regulated during chondrogenesis. It now seems quite likely that these distinct genes of cartilage matrix macromolecules are coordinately regulated during limb cartilage differentiation, which enables these matrix components to form the very precise temporal and spatial arrangement necessary for the cartilage structure formation.

We are grateful to Drs Y. Ninomiya and B. R. Olsen for generously providing cDNA probes.

Received for publication 13 June 1988; and in revised form 30 June 1988

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