Regulation of the Expression of Genes Encoding Types I, II, and III Collagen during Chick Embryonic Development*

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During the embryonic development of the chicken, stimulation of production of collagen-enriched tissue such as bone matrix, cartilage matrix, and skin dermis occurs between day 7 and day 15. We have examined the levels of the RNAs encoding the interstitial collagens (types I, II, and III) to determine if this developmental progress is associated with increased accumulation of collagen RNA. Using cell-free translation and DNA:RNA hybridization techniques, we have detected a coordinate enhancement of ~10-fold in the steady state levels of whole embryo RNAs encoding the \( \alpha 1(1) \), \( \alpha 2(1) \), \( \alpha 1(II) \), and \( \alpha 1(III) \) collagens between 5 and 10 days of chick embryonic development. The developmental pattern of expression of these collagen genes in whole embryos is in marked contrast to that of two noncollagenous proteins; cellular fibronectin and \( \beta \)-actin, whose RNA levels were not found to change dramatically during early chicken development. In addition, we have observed that at least 3 of the 4 collagen genes examined were expressed at low levels beginning between 1–2 days. Both 5'- and 3'-specific \( \alpha 2(1) \) collagen gene DNA probes hybridized to early stage and late stage chick embryo RNAs of identical sizes. DNase I-hypersensitive sites have been detected near the 5' end of the \( \alpha 2(1) \) collagen gene in chromatin isolated from both 2-day and 5-day embryos, representing developmental time points well before and at the threshold of the onset of enhanced collagen RNA synthesis, respectively. These results suggest that the same gene is expressed in early and late chick embryos to yield \( \alpha 2(1) \) collagen RNAs of similar structure.

The collagens are a broad family of structural proteins which function as an extracellular framework in eukaryotic organisms. They are characterized by a unique protein conformation which consists of three \( \alpha \) polypeptide chains in a triple helix. At least nine genetically distinct collagen \( \alpha \) chains have been identified in vertebrates, with the likelihood that many more exist (1, 2). Type I collagen is the most prevalent, found in bone, tendon, and skin; type II collagen is predominantly localized in cartilage; type III collagen is located in blood vessels, uterus, and fetal skin; and types IV and V are for the most part found in various basement membranes (1, 2).

The collagens have an extremely important role during embryonic development (3). Collagen levels measured indirectly by quantitation of hydroxyproline in developing *Xenopus laevis* embryos were found to increase more than 100-fold between gastrula and the feeding tadpole (4). A *Drosophila* collagen gene has been shown to be expressed in a developmentally regulated manner (5). In the chick embryo, the collagens are produced in specific combinations during the growth and development of major connective tissues such as bone, cartilage, and parts of the skin. In addition, the collagens comprise a significant part of the connective tissue found in developing organs such as heart, lung, liver, and eye (6, 7).

Collagen gene expression is altered in a variety of human pathologic states (2, 8). In addition, cultured cells exhibit significant qualitative and quantitative changes in collagen production in response to exposure to various factors, including bromodeoxyuridine, ascorbic acid, chick embryo extracts, cAMP, Ca++, and chemical or viral transformation (2). Treatment of cells with agents such as these results in a modulation of collagen synthesis (called “switching”) between types I, II, and III. The significance of collagen switching remains unclear.

The number of copies of each collagen gene has been extensively investigated, and there is considerable evidence that each member of the collagen gene family is represented in the vertebrate genome as a single copy gene. The human \( \alpha 2(1) \) collagen gene copy number was found to be one by DNA dot blot hybridization (9), and it has been localized to a single chromosomal site (10). In addition, a single recessive mutation is thought to be responsible for the absence of \( \alpha 2(1) \) collagen in a patient with osteogenesis imperfecta (11). In the chicken, the pattern of \( \alpha 2(1) \) collagen-specific genomic DNA fragments generated by digestion with numerous restriction enzymes matches the pattern of genomic phase DNA and indicates a single copy number (12). Also, we have successfully isolated the entire chick \( \alpha 2(1) \) and the \( \alpha 1(III) \) collagen genes by “walking” up the gene without encountering other genes (13, 14).

Recent evidence obtained from comparison of the structure of collagen proteins (2, 15) as well as comparison of cloned DNA sequences coding for \( \alpha 2(1) \), \( \alpha 1(II) \), and \( \alpha 1(III) \) chick collagen suggests that these genes encoding the chicken collagens constitute a multigene family with common evolutionary origins (14, 16–19). Because the chick collagen genes are represented as single copies in the genome, it seemed possible to analyze their regulation during development. We decided to examine the behavior of this multigene family during embryonic development by determining qualitative and quantitative changes in whole embryo RNAs coding for the interstitial (types I, II, and III) collagens. Our goal was to deter-

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mine the relationship between the appearance of these RNA species, the synthesis of collagen proteins, and the development of collagen-enriched organs. In addition, we compared the regulation of collagen gene activity during development to other two genes, one encoding another extracellular protein (cellular fibronectin) and a second coding for an abundant intracellular structural protein (β-actin).

**MATERIALS AND METHODS**

Embryos and Cells—Chick embryos (Gallus gallus), incubated at 38 °C, were obtained from Truslow Farms, Inc. (Chestertown, MD) at appropriate developmental time points. Approximately 50 24-h embryos (Hamburger and Hamilton stage 6–8 [20]) were collected for RNA isolation. The embryonic shield region was used while discarding the area opaca. About 30 48-h embryos (stage 12–13) were used for RNA isolation, again excluding the area opaca. In addition, the covering amniotic sac membrane was removed from older embryos before use. For later stages, between one and 20 animals were collected. In all cases, embryos were quick frozen in dry ice until RNA was isolated. CEF were cultured from 10-day embryos at 38 °C as described previously (21).

RNA Isolation—Total RNA was prepared by solubilization of whole embryonic tissue in 8 M guanidine HCl, followed by precipitation of RNA in 0.5 volumes of −20 °C ethanol as described elsewhere (21, 22). The quality of the RNA was established by 260–280 absorbance, ethidium bromide staining of RNA electrophoresed on agarose gels, and translatability in a cell-free protein synthesizing system (see below). The RNA was obtained from nuclei isolated according to Groudine et al. (23). The nuclei were lysed in 4% Sarkosyl and the RNA centrifuged through a CsCl gradient according to Glisin et al. (24).

Cell-free Translation and Protein Gel Electrophoresis—Cell-free translations were performed using rabbit reticulocyte lysate (25) and 35S-labeled methionine obtained from Amersham. Reactions, routinely containing 1 μg of total RNA and 6 μl of lysate in a final volume of 8 μl, were incubated at 30 °C for 1 h and then subjected to ethanol precipitation. Some reaction mixtures were treated with collagenase ( Worthington) before precipitation for 2 min at 37 °C in 50 mM CaCl2 and 62.5 mM N-ethylmaleimide.

Precipitated proteins were dissolved in sample buffer and fractionated on a 3% over 9% polyacrylamide-SDS gel as previously described (21, 26). Radioactive polypeptides were visualized by standard fluorographic techniques.

Electrophoretic Fractionation and Blotting of RNA—The gel system used was a modification of Goldberg (28). RNAs (3 μg/well) were heat denatured at 65 °C for 5 min in 1 × Mops buffer (19.5 mM morpholinopropanesulfonic acid (pH 7.0), 5 mM Na acetate, 1 mM EDTA) in the presence of 33% formamide and 4.95% formaldehyde. RNAs were fractionated on 1% agarose containing 1 × Mops buffer and 6.2% formaldehyde at 65–70 °C for 3.5–6 h. Running buffer was 1 × Mops buffer.

The resulting gel was stained in ethidium bromide in running buffer and transferred to nitrocellulose as described by Thomas (29). The blots were vacuum dried and hybridized to appropriate DNA probes (see below). RNA standards included Erheria coli rRNA (1540 and 2900 bases) and chicken rRNA (1900 and 4100 bases). λ standards in formaldehyde gels were found to be inaccurate and were not used.

Dot blots were performed according to Thomas (29), where 4 μg of RNA were spotted per sample.

Isolation and Blotting of DNA—Nuclei were isolated from 2-day (stage 11) and 5-day (stage 26–27) chick embryos. Embryos were collected on ice, washed, and homogenized in an isotonic buffer as described by Wu et al. (30). The nuclei were centrifuged through a 1.5 M sucrose cushion at 100,000 × g for 30 min, and suspended in DNase I digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol, 0.05 mM CaCl2, 3 mM MgCl2, and 0.25 mM sucrose) to give a DNA concentration of ~200 μg/ml (31). These nuclei were divided into 100-μl reactions and digested with increasing amounts of DNase I (Boehringer-Mannheim) for 3 min at 23 °C. For each series of DNase digestions, the number of nuclei used was equivalent to 60 2-day embryos and four 5-day embryos.

DNA was isolated according to Wu et al. (30). The DNA was cleaned electrophoresed on a 2% agarose gel in 1 × TBE buffer, transferred to nitrocellulose filters (31). The hybridization and washing conditions are described elsewhere (12). To detect the hypersensitive site upstream of the α2(I) collagen gene, a 300-bp HindIII-EcoRI, nick translated (32), 3P-labeled fragment was isolated from a subclone of xccl-323 which contains genomic DNA coding for part of the α2(I) collagen gene (10).

Plasmid DNA—To probe for specific RNA sequences, various plasmid DNAs were utilized. These include cloned chicken cDNA sequences specific for α(I) collagen (pCOL 3 (33)), α2(I) collagen (pCOL 1 (34)), α1(I) collagen (pCAR 1 (19)), and β-actin (pA2 (35)). In addition, we have taken advantage of recently isolated genomic DNA fragments coding for chicken cellular fibronectin (36) and chicken α1(III) collagen (14). Plasmid pCAR I was a gift from B. Upholt (University of Chicago). Plasmid pA2 was generously supplied by D. Cleveland (Johns Hopkins University). The chicken fibronectin (pCAR II HindIII-EcoRI) and α1(II1) collagen (pA2 HindIII EcoRI) were kindly provided by H. Hirsh (LMB, National Institutes of Health), and the 400-bp Xcl-323 DNA fragment from M. Madryz and Y. Yamada (LMB, National Institutes of Health).

Labeling of DNA and Hybridization—For hybridization probes, either whole plasmid DNA or DNA fragments were utilized. Plasmids were prepared using chloroamphenicol amplification and CsCl equilibrium centrifugation techniques. Restriction endonuclease digestions were as described by the manufacturer (New England Biolabs). DNA fragments separated on polyacrylamide gels were extracted according to Maxam and Gilbert (37), and fragments separated on agarose gels were recovered by electrophoretic elution.

DNA was labeled with [α-32P]deoxyribonucleotides by the method used by Maniatis et al. (32). The nick-translated probe was separated from unincorporated nucleotides by G-100 Sephadex column chromatography.

Prehybridization, hybridization, and washing conditions were as described by Thomas (29), except that buffers included 0.1% SDS, and hybridization was for 18–36 h at 42–45 °C. Because of observed cross-hybridization between pCAR I plasmid DNA (containing sequences coding for chick α(I) collagen) and chicken α(I) collagen RNA, hybridization with the pCAR I probe was performed at 52 °C. Stringent washing conditions were employed in all cases to remove background radioactivity (0.2 × SSC, 0.1% SDS at 50 °C for 15 min twice). Radioactive bands were visualized using Dupont light-plus intensification screens.

**RESULTS**

Changes in Translatable RNA during Chick Development

Initially, we determined how the population of translatable RNAs changed during the embryonic development of the chicken. Total RNA was isolated from 1-, 5-, 10-, and 15-day chick whole embryos using 8 M guanidine HCl solubilization and half-volume ethanol precipitation. These RNAs were translated in a reticulocyte lysate cell-free translation system, and the resulting radioactive polypeptides were fractionated by SDS-polyacrylamide gel electrophoresis. The autoradiographic results are displayed in Fig. 1. There are numerous significant alterations in the levels of translatable RNAs as development proceeds (Fig. 1, lanes a, c, e, and g). One of the most striking increases is that of translatable RNA coding for the collagen, including the α1 and α2 polypeptide chains of type I procollagen (Fig. 1, lanes d and f). These proteins were not detectable at day 1 but became prominent by days 10 and 15. The bands designated as α1(I) and α2(I) procollagen are tentatively identified because their apparent molecular weights are ~190K and 155K, respectively, in agreement with Adams et al. (21). Both bands were sensitive to collagenase (Fig. 1, lanes b, d, f, and h) and were enriched in a cultured cell type (CEF) known to contain relatively high levels of type
Development of chick embryos at various developmental time points (in days at the top of the autoradiogram). 35S-labeled, in vitro-synthesized polypeptides were prepared for electrophoresis directly (-) or after treatment with bacterial collagenase (+), fractionated on a SDS-9% polyacrylamide gel, and visualized by fluorography. Fn, migration of a chick fibronectin standard; T, migration of unseparated a and b tubulin standards; A, location of chick actin; a1C, location of chick pro-a1(I) collagen; a2C, location of chick pro-a2(I) collagen; molecular weight markers (in kDa) are shown at left; CEF, chick embryo fibroblast total RNA; bk, lane k depicts the endogenous (RNA) translation products.

Developmental Regulation of the Expression of the Collagen Genes

Type I Collagen—Early studies have shown that collagen levels are dramatically elevated during chicken development (7, 38–40). In particular, an enhancement in collagen synthesis has been detected between 7 and 15 days of development (39). This finding correlates well with the development of the major collagen-containing tissues. Synthesis and ossification of bone matrix in the chick skeleton proceeds rapidly between 9 and 14 days, and the onset of chondrification in vertebrae, the skull, and the limbs is between 6 and 8 days (6).

To determine how the level of type I collagen RNA varies during development, total RNA was isolated from 1-, 2-, 3-, 4-, 5-, 10-, and 15-day whole chick embryos, spotted onto nitrocellulose paper (4 µg/spot), and probed with type I collagen-specific 32P-labeled cloned DNA. This procedure allowed us to quantitate changes in total hybridizable type I collagen RNA. The plasmid pCOL 3 containing cDNA sequences coding for the COOH terminus of a1(I) collagen (33) was 32P-labeled by nick-translation and hybridized to the dot blots. The hybridizable radioactivity was quantitated by either microdensitometry or direct liquid scintillation counting, and expressed as a percentage of hybridizable day 15 RNA. The procedure was performed using nick-translated plasmid pCOL 1 containing cDNA coding for the COOH terminus of a2(I) collagen (34). Fig. 2 shows that little change occurs in either RNA during the first 5 days of development, but thereafter a dramatic enhancement (greater than 10-fold) in the levels of both a1(I) and a2(I) collagen RNA is observed. These results are consistent with the cell-free translation data (Fig. 1) and are generally in agreement with the developmental progress of tissues abundant in type I collagen such as bone and skin. They suggest that the synthetic rate of collagen is dependent on the concentration of its RNA, in agreement with the results of Moen et al. (41), who found a direct correlation between the levels of procollagen and procollagen mRNA in developing chick embryo calvaria.

The dot blot procedure is limited, however, in that it offers no structural information about the hybridizable RNA sequences (i.e. large precursor RNA, mature mRNA, and degradation products). To overcome this limitation, whole embryo RNAs isolated from representative developmental time points were electrophoretically fractionated on 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with 32P-labeled a1(I)- and a2(I)-specific DNA probes.

Fig. 3 shows that the a1(I) collagen probe (plasmid pCOL...
were capable of hybridizing to pCa2PRO-3 DNA, a plasmid to hybridize to pCOL1 DNA sequences (Fig. 4). To elucidate transcription (17, 43). The developmental pattern of expression of the genes containing pCa2PRO-3 DNA sequences was containing nontranslated leader sequences at the extreme end of the \( \alpha2(1) \) collagen gene including the start site of the 3' end of \( \alpha2(1) \) collagen gene as evidenced by their ability to hybridize to pCOL1 DNA sequences (Table I). They both accumulate slowly up to day 5 and thereafter at a much greater rate.

A similar RNA blot was hybridized to the cDNA probe coding for the 3' end of \( \alpha2(1) \) collagen RNA (pCOL1), and the results are shown in Fig. 4. Two RNA species of 5.1 and 5.7 kilobases are visible in total RNA from chicken embryos as well as in CEF (Fig. 4, lanes i and j') which is in agreement with previously published results on CEF collagen RNA (42). The \( \alpha2(1) \) collagen RNA accumulates in a manner similar to that of \( \alpha1(1) \) RNA in that a dramatic enhancement in the level of both RNA species is observed between day 5 and day 10. In addition, both the 5.1- and 5.7-kilobase RNA species were detectable in 24-h embryos (Fig. 4, lanes a and a'), the earliest time examined.

These early embryonic RNAs have clear homology to the 3' end of the \( \alpha2(1) \) collagen gene as evidenced by their ability to hybridize to pCOL1 DNA sequences (Fig. 4). To elucidate further the nature of these early RNAs, we determined if they were capable of hybridizing to pCa2PRO-3 DNA, a plasmid containing nontranslated leader sequences at the extreme 3' end of the \( \alpha2(1) \) collagen gene including the start site of transcription (17, 43). The developmental pattern of expression of the genes containing pCa2PRO-3 DNA sequences was identical to that found using pCOL1 as a probe (data not shown), proving that not only the 3' end but also the 5' nontranslated end of \( \alpha2(1) \) collagen RNA are similar (if not identical) in early and late stage embryos. These results suggest that the stable RNA species found in day 1-5 embryos are essentially the same as those located in day 15 embryos and argues against the existence of separate embryonic collagen genes.

In other genes, the temporal appearance of DNase I hypersensitive sites correlates well with the onset of transcriptional activity during embryogenesis (i.e. the globin gene during chick development (44, 45)). If the same gene codes for both early and late embryonic collagen RNAs, then one might expect to see similar patterns of DNase I sensitivity in chromatin from very early as well as late stage embryos. We therefore examined the \( \alpha2(1) \) collagen gene in chromatin for hypersensitive sites by treating nuclei isolated from 2-day embryos (many days before stimulation of \( \alpha2(1) \) collagen RNA synthesis) and from 5-day embryos (at the threshold of the observed increase) with various amounts of DNase I, and then probing DNA purified from each for the existence of prominent digestion products. One such hypersensitive site has been mapped ~200 bp upstream of the \( \alpha2(1) \) collagen gene in CEF chromatin which appears to be correlated with gene activity. Fig. 5 reveals that chromatin of 2- and 5-day embryos contains DNase I hypersensitive sites located in the same region as previously found in CEF chromatin (lanes c, f, and g, closed arrows). In addition, a second less sensitive site is seen in embryonic chromatin from each stage (Fig. 5A, lanes c and f, open arrow). This site maps in the first intron of the \( \alpha2(1) \) collagen gene (Fig. 5B). A similar pattern has been demonstrated in the chromatin from Rous sarcoma virus-transformed CEF which also express low levels of \( \alpha2(1) \) collagen (data not shown). These results indicate that the chromatin structure of \( \alpha2(1) \) collagen gene is in a configuration which would permit transcription of the gene. Taken together with the RNA data, our findings suggest that the \( \alpha2(1) \) collagen gene is active not only at 5 days of development but even as early as 2 days, further supporting the notion that...

### Table I

**Comparison of the accumulation of high versus low molecular weight type I collagen RNA species**

<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Days of development</th>
<th>High molecular weight RNA</th>
<th>Low molecular weight RNA</th>
<th>High/low ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha1(1) )</td>
<td>1</td>
<td>ND1</td>
<td>10'</td>
<td>ND</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>2</td>
<td>5</td>
<td>50</td>
<td>0.10</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>3</td>
<td>11</td>
<td>61</td>
<td>0.18</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>4</td>
<td>14</td>
<td>102</td>
<td>0.14</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>5</td>
<td>22</td>
<td>111</td>
<td>0.20</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>10</td>
<td>390</td>
<td>1490</td>
<td>0.26</td>
</tr>
<tr>
<td>( \alpha1(1) )</td>
<td>15</td>
<td>730</td>
<td>3350</td>
<td>0.22</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>1</td>
<td>13</td>
<td>17</td>
<td>0.76</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>2</td>
<td>25</td>
<td>18</td>
<td>1.38</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>3</td>
<td>36</td>
<td>32</td>
<td>0.81</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>4</td>
<td>48</td>
<td>53</td>
<td>0.91</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>5</td>
<td>57</td>
<td>73</td>
<td>0.78</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>7</td>
<td>165</td>
<td>218</td>
<td>0.76</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>10</td>
<td>320</td>
<td>570</td>
<td>0.56</td>
</tr>
<tr>
<td>( \alpha2(1) )</td>
<td>15</td>
<td>570</td>
<td>1460</td>
<td>0.39</td>
</tr>
</tbody>
</table>

1 High and low molecular weight species of \( \alpha1(1) \) RNA are 7.1 and 5.0 kb, respectively.
2 ND, not detectable.
3 Levels of RNA were quantitated by microdensitometric scanning of autoradiograms (i.e. Figs. 3 and 4) and subsequent determination of area under curves. Levels are expressed in arbitrary units. Numbers obtained for \( \alpha1(1) \) RNA have no direct relationship to those for \( \alpha2(1) \) RNA.
4 High and low molecular weight species of \( \alpha2(1) \) RNA are 5.7 and 5.1 kb, respectively.

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2 C. McKeon, I. Pastan, and B. de Crombrugge, manuscript submitted.
Type II and Type III Collagen—Having established the developmental pattern of expression of the type I collagen gene, we wanted to determine if other genes in the collagen family were regulated similarly. Embryonic RNAs from appropriate developmental stages were fractionated and transferred to nitrocellulose as previously described and then hybridized to either a $^{32}P$-labeled α1(II) collagen cDNA probe (plasmid pCAR I, generously provided by B. Upholt, University of Chicago (19)) or a $^{32}P$-labeled α1(III) collagen DNA probe (a 400-base pair Rsal nonrepetitive genomic DNA fragment (14)).

Fig. 6 shows the developmental pattern of expression of the α1(II) collagen gene. One major RNA species of 5.3 kilobases is detected in chick embryos. In a manner similar to type I collagen, type II collagen RNA appears early in development (48 h, lane b) and increases markedly between day 5 and 10. The level at day 15 seems to be somewhat reduced. This behavior is in general agreement with the known biology of cartilage development. Cartilage is laid down during chondrogenesis, beginning at 6–8 days, but then is replaced by bone at later stages (6). That the pCAR I probe is specific only for type II RNA is confirmed by its ability to hybridize to sternum RNA, but not to crop or CEF total RNA (Fig. 6, lanes j, h, and i, respectively).

Fig. 7 reveals that α1(III) collagen RNA accumulates in a manner similar to that seen for the other collagen RNAs. The major difference is that very little type III collagen RNA is detected between 1 and 5 days. On day 10 and 15, however, a substantial amount of type III collagen RNA is found in the form of a ~6.5-kilobase species. The genomic DNA probe also hybridizes to a smaller 2.6-kilobase RNA (Fig. 7, lanes f and i).
and g) of unknown origin which appears to be coordinately regulated. The fact that \( \alpha_1(III) \) collagen RNA represents a higher percentage of whole embryo RNA in days 10 and 15 relative to earlier stages is probably due to the continuing differentiation of embryonic skin from condensation of mesenchyme cells, giving rise to dermal connective tissue and collagen fibers at about day 12 (6, 46). More specifically, Vinson and Seyer (40) have found significant quantities of \( \alpha_1(III) \) collagen in chick skin between 7 and 12 days of development, again in agreement with our RNA data.

In summary, all of the collagen genes examined are expressed in a similar developmental pattern in which collagen RNAs represent a significantly higher percentage of whole embryo RNA in late embryonic life than in early. To insure that the observed increase in steady state collagen RNA levels associated with late stage embryos was not an artifact of the RNA preparations, we next compared the regulation of collagen gene expression to that of two noncollagenous proteins i.e. cellular fibronectin, an extracellular protein known to have an affinity for the collagens (47) and \( \beta \)-actin, an abundant intracellular structural protein.

**Developmental Regulation of the Expression of Genes Encoding Fibronectin and Actin**

**Fibronectin**—Fibronectin, a component of many basement membranes and of loose connective tissue matrix, has been detected in the early chick embryo by immunofluorescent techniques (48) and may play an important role during development (49). DNA:RNA hybridization was performed using a 2.0-kilobase pair nonrepetitive genomic DNA fragment subcloned from the 3' end of the cloned chick cellular fibronectin gene (36) as probe to determine the pattern of fibronectin gene expression during development. Fig. 8 reveals that in marked contrast to the results obtained for the collagens, the level of whole embryo fibronectin-specific RNA, represented by one major 7.5-kilobase species, remains relatively constant throughout development, including day 1. These data are consistent with published reports in which fibronectin proteins were detected in the first day of chick development (48).

Furthermore, we have found that the amount of fibronectin RNA/\( \mu \)g total RNA is higher in CEF than in any of the embryos tested (Fig. 7, lane h).

**\( \beta \)-Actin**—The results of the cell-free translation data (Fig. 1) suggest that the levels of \( \beta \)-actin mRNA do not change to the same extent during chick development as the levels of collagen mRNA. To test this and to compare actin gene expression to that of the collagens, fractionated whole embryo RNAs immobilized on nitrocellulose were hybridized with a \( ^{32}P \)-labeled DNA probe specific for the 3' nontranslated region of one chicken \( \beta \)-actin gene (the 550-base pair insert from the plasmid pA2, a gift from D. Cleveland, Johns Hopkins University (35)). The results are shown in Fig. 9. A short autoradiographic exposure reveals that, like fibronectin, \( \beta \)-actin mRNA (2.0 kilobases) does not dramatically change in whole embryos during development; however, the level of this major actin species may be somewhat reduced in day 15 embryos, and is about 2-fold higher at 24 h than at any developmental time thereafter. That actin RNAs are synthesized at significant levels during very early development has previously been demonstrated in sea urchin and *Drosophila* embryos (22, 50, 51).

In addition to the 2.0-kilobase mature mRNA (35), we have detected at extremely low levels several other larger RNA species which also fail to exhibit any developmentally specific changes (Fig. 9, lane i). There are two very large RNAs of 7.0 and 7.9 kilobases and also intermediate sized RNAs of about 4.3, 3.9, 3.4, 3.1, and 2.8 kilobases. Interestingly, there is a remarkable correlation between the differences in the sizes between the intermediate sized RNAs (400, 500, 300, and 300 bases) and the length of chicken \( \beta \)-actin gene introns determined by DNA sequence analysis (319, 524, 306, and 354 base pairs). The possibility that these RNA species represent intermediates in the processing of a larger \( \beta \)-actin precursor RNA remains to be proven.

**DISCUSSION**

We have demonstrated that RNAs coding for types I, II, and III collagen accumulate dramatically in the whole chick embryo between 5 and 15 days of development. This is in accordance with the finding that there is a significant elevation in levels of collagen during this same period of time (6, 7, 38–40). In addition, this striking increase parallels the developmental progress of collagen-enriched tissues, type I collagen in embryonic bone matrix, type II in precartilagenous matrix, and type III in the dermis of embryonic skin. These studies only answer questions relating to changes in steady state collagen, actin, and fibronectin RNA levels in the whole chick embryo. We assume, of course, that in different cell types in the developing embryo the genes are differentially regulated. *In situ* hybridization would directly address this problem.

The observation that the collagen gene family is apparently regulated coordinately is somewhat surprising. Different embryonic cells must be responsible for synthesizing specific dominant collagen types (I, II, or III, in osteoblasts, chon-
droblasts, and embryonic dermal fibroblast cells, respectively). All these cell types are derived from local condensation of mesenchymal cells, and the process of differentiation to osteoblasts or chondroblasts must be rapidly accompanied by stimulation of specific collagen production (6, 7). A question that arises concerns the nature of the signals that trigger the differentiation and/or collagen synthesis. Because the many types of collagen RNAs accumulate in a temporally coordinate manner (see *Results*), it is possible that a single factor or event spreading throughout the chicken embryo at one developmental time point is responsible for this differentiation and/or enhanced collagen synthesis. Striking similarities have been discovered in the structure of the DNA surrounding the translation initiation region of the al(I), a2(I), and a1(II) chicken collagen genes, suggesting that common regulatory mechanisms may exist. Alternatively, the temporal similarity in production of the various collagen RNAs could be coincidental, while the various cell types involved could rely on very different environmental factors as signals to begin synthesizing the appropriate type of collagen.

A point that deserves further consideration concerns the role of collagen in the early chick embryo (3, 52). We have found detectable levels of a1(I) and a1(II) collagen RNA by 48 h of development, and a2(I) collagen RNA by 24 h. These data are consistent with reports that type I collagen was detected in the dermatome-myotome plate at stage 17, and in the eye at stage 19, while type II was found in the notochord and spinal cord epithelium at stage 15, and in parts of the eye at stage 20 (53–56). In addition, quantitation of proline hydroxylation levels suggests that collagen was present in chick heart at stages 9–11 (57). However, the low levels of collagen RNA at 1–4 days stands in sharp contrast to the relatively high quantities of fibronectin RNA (Fig. 8), b-actin RNA (Fig. 9), or b-tubulin RNA found in 24-h embryos. The early collagen RNAs are probably not part of a maternal messenger RNA pool because most collagen types are represented by detectable levels of RNA at 48 h, but not at 24 h of development. The one exception (a2(I) collagen RNA), which appears in the earliest development time point examined (24 h), is not a likely candidate for a maternal messenger because of the existence of a DNase I hypersensitive site in the a2(I) collagen-specific chromatin of stage 11 embryos (Fig. 5). This finding suggests that the a2(I) collagen gene is active early in development, and therefore the detectable RNAs may be synthesized de novo in the embryo.

The nature of the early collagen RNAs is not known. Because the available data strongly support the idea that the genes encoding the various collagen types exist as single copies in the genome (see "Introduction"), we felt it likely that early embryonic RNAs did not originate from separate embryonic genes. In the current studies, we found that early a2(I) collagen RNAs are probably not strictly embryonic species because they are the same size as, and contain sequences homologous to the 5' and 3' ends of, late embryo a2(I) collagen RNA. In addition, DNase I hypersensitive sites were detected in a2(I) collagen chromatin in early as well as late embryos (Fig. 5). Although the same gene seems to be active throughout embryonic life, we cannot rule out the possibility that early a2(I) collagen RNAs and/or their protein counterparts may be processed differently than late RNAs. The resulting early embryonal connective tissue collagens may play very different roles in the developing chick. Collagens, helping to form the early embryo extracellular matrix, might be sequestered into embryonic spaces where they could influence cells surrounding such spaces by binding to putative collagen surface receptors (3). In this way, collagen may act as an embryonic inducer. There is evidence that the collagens play a role in binding morphogenetic factors that promote differentiation of embryonic tissues (3). Reddi (52) proposed that the collagen matrix may play an important role in the embryo by conveying local positional information. It is clear that collagen and fibronectin have a critical role in the adhesion and interaction between cells in culture (47, 58). Collagen and fibronectin might function in this capacity in the early chick embryo where cells are continuously moving and reorienting themselves.

Another interesting feature of the regulation of the collagens involves the behavior of the two species of a2(I) collagen RNA. We have found that there is a change in the relative ratios of the higher molecular weight (5.7 kilobases) RNA species compared to the lower (5.1 kilobases) species (Table I). During the first 5 days of development, the band representing the 5.7-kilobase RNA species is at least as intense as the 5.1-kilobase band; however, by 10 and 15 days, the 5.1-kilobase band clearly becomes predominant (Fig. 4). In contrast, the two a1(I) RNA species seem to accumulate in a parallel fashion (Figs. 3 and Table I). In order to understand the significance of the differential temporal appearance of the two a2(I) collagen RNA species, it may be necessary to clarify the relationship between them. Myers et al.7 have shown that the single copy human a2(I) collagen gene is used as a template for the synthesis of both a high and a low molecular weight species of collagen RNA. The two RNAs differ only at the 3' site of polyadenylation. It is possible that the same mechanism is responsible for the genetion of two chick a2(I) collagen RNAs. We have shown that both species of a2(I) RNA are present in the nucleus and the cytoplasm of CEF (Fig. 4), suggesting that the larger is not a precursor species. It is conceivable that the two chicken a2(I) collagen RNA species accumulate in a spatially and temporally different manner during development. Hybridization in situ may help determine if tissue-specific alterations in collagen RNA synthesis exist to account for the qualitative and quantitative changes in whole embryo RNA observed during embryonic chick development.

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**Footnotes:**

Developmental Expression of Chick Collagen Genes

Quantitative Analysis of Prenatal Development, John Wiley and Sons, New York


Regulation of the expression of genes encoding types I, II, and III collagen during chick embryonic development.
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