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Expression and Distribution of Two Alternatively Spliced Transcripts From the Chicken α2(VI) Collagen Gene

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Abstract Two types of mRNA molecules with different 3′ ends are transcribed from the chicken α2(VI) collagen gene. The major splice variant encodes a polypeptide with a von Willebrand factor A domain at its carboxyl terminus. In the minor splice variant, this A domain is replaced by a novel motif which reveals some similarity to a fibronectin type III repeat. In situ hybridization experiments demonstrate that the major transcript is ubiquitously expressed. Substantial amounts are found in skeletal and cardiac muscle, gizzard, skin, tendon, liver, the wall of blood vessels, and the connective tissue of peripheral nerves. In contrast, the minor transcript is expressed at a very low level and can hardly be detected in any tissue by in situ hybridization. Only the aortic wall contains a considerable amount of this splice variant. However, no difference is observed by Northern blotting and the polymerase chain reaction in the ratio of the two transcripts when aorta and the other tissues are compared. Thus, the minor splice variant is not expressed in a tissue specific manner and, consequently, it is unlikely that it plays a tissue specific role. It might rather serve a general function in the structure and assembly of type VI collagen microfibrils.

Key words: alternative splicing, collagen VI, extracellular matrix, in situ hybridization, tissue distribution

The large family of collagens contributes in many ways to the integrity and tissue specific structure of the extracellular matrix. Some of its members constitute the insoluble scaffold of the interstitial collagen fibrils, while others connect these fibrils with further components of the extracellular matrix or with the cell surface [for review, see Prockop and Kivirikko, 1995; Pihlajaniemi and Rehn, 1995]. Collagen VI represents such a connecting element [Timpl and Chu, 1994]. This protein is thought to integrate the interstitial collagen fibrils in the extracellular matrix by the formation of an independent microfibrillar network that binds to the major fibrillar structures. A direct interaction of collagen VI with collagen I has in fact been demonstrated in vitro [Bonaldo et al., 1990]. Furthermore, collagen VI is known to bind to non-fibrillar collagens, to various glycosaminoglycans and proteoglycans as well as to von Willebrand factor [Timpl and Chu, 1994]. In vitro, it promotes stable adhesion and spreading of fibroblasts. Several cell surface receptors have been described for this collagen, including membrane-associated proteoglycans and integrins of the β1 family [Timpl and Chu, 1994].

The diversity of interactions displayed by collagen VI is reflected at the molecular level by the complex modular structure of the protein. Collagen VI is a heterotrimer made up of three genetically distinct subunits, the α1(VI), α2(VI), and α3(VI) collagen chains. The primary structure of all three subunits has been elucidated by cDNA cloning for the chicken [Koller et al., 1989; Bonaldo and Colombatti, 1989; Bonaldo et al., 1989, 1990] and the human protein [Chu et al., 1988, 1989, 1990]. These studies revealed a hybrid molecule containing a short triple helix flanked by large globular domains. The globular domains are composed of several homologous repeats, each repeat comprising about 200 amino

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acid residues. These repeats show a striking similarity to the collagen-binding motifs identified in von Willebrand factor, cartilage matrix protein, and some integrin receptors [Colombatti and Bonaldo, 1991]. The α1(VI) and the α2(VI) collagen chains harbor three of these von Willebrand factor A (VWFA) motifs, one in the amino terminal and two in the carboxyl terminal non-collagenous domain. The α3(VI) collagen chain contains 12 VWFA repeats, ten at the amino terminus and two at the carboxyl terminus. In the genome, most of these VWFA domains are encoded by a single exon of 600 bp, whereas the triple helical region is encoded by a large number of very short exons [Doliana et al., 1990; Wälchli et al., 1992; Saitta et al., 1990; Stokes et al., 1991].

Further complexity to the structure of collagen VI stems from the existence of several different mRNA isoforms that arise by alternative splicing. In the α3(VI) collagen gene, at least three exons coding for VWFA repeats are subject to alternative splicing [Doliana et al., 1990; Stokes et al., 1991; Zanussi et al., 1992]. Exon skipping appears to generate several distinct α3(VI) collagen polypeptides which contain a variable number of VWFA repeats at their amino terminal end. Interestingly, the splicing pattern of these subdomains differs between normal fibroblasts and some human tumor cell lines [Stokes et al., 1991].

So far there is no evidence for alternative splicing of the α1(VI) collagen transcript. In the case of the α2(VI) collagen gene, however, two different splice variants can be predicted from the DNA sequences of the chicken and the human gene. These may be generated by alternative usage of the last two exons, termed 28A and 28B, in a mutually exclusive manner (Fig. 1). The major form of the α2(VI) collagen transcript contains the sequence of exon 28B which encodes the third VWFA domain. If exon 28A is included in the final transcript instead of exon 28B, this VWFA domain is replaced by a shorter element that reveals some similarity to a fibronectin type III repeat [Saitta et al., 1990; Hayman et al., 1991]. In the chicken, the potential usage of exon 28A has not yet been demonstrated at the mRNA level. In man, the corresponding mRNA was found on Northern blots, but its abundance was extremely low so that no further attempts were made to study its distribution. The analysis of the human gene suggested the existence of even a third variant with a

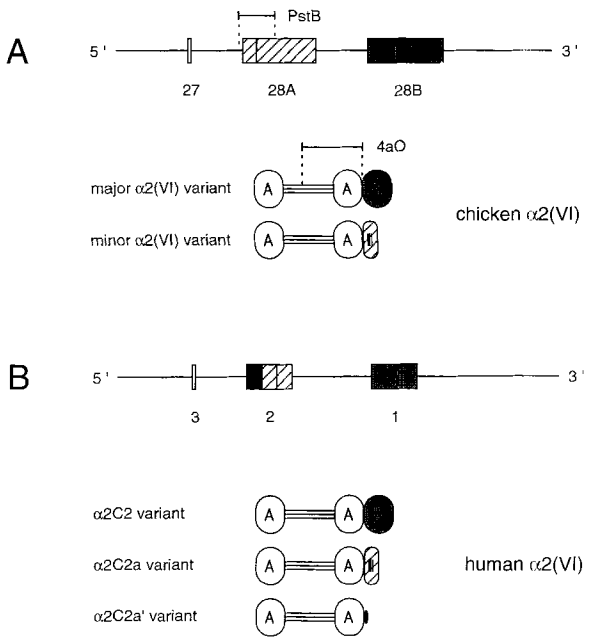

Fig. 1. Schematic drawing of the α2(VI) collagen isoforms and of the genomic region encoding them. The chicken isoforms are displayed in **A**, the human isoforms in **B**. The VWFA repeats and the fibronectin type III motifs are shown by circles, the collagenous region is indicated by three horizontal lines. At the top, the three exons encoding the carboxyl terminal part of the polypeptides are shown and numbered according to original publications. Exons that are subject to alternative splicing are indicated by grey, hatched, and black boxes, respectively. Translation stop codons are marked by vertical lines within the exons. The relative positions of the two DNA probes used in this study (PstB, 4aO) are given by horizontal bars.

truncated carboxyl terminal domain [Saitta et al., 1990]. This variant could not be detected on Northern blots and there is no evidence for the existence of a similarly truncated variant in the chicken.

It may be expected that the replacement of a VWFA domain by a fibronectin type III module would drastically alter the biological properties of the resulting polypeptide. Hence, the events that lead to differentially spliced products might be tightly controlled in a tissue specific manner. The tissue distribution of the α3(VI) collagen isoforms has been investigated in some detail and a tissue specific expression of at least one variant has been reported [Doliana et al., 1990]. In contrast, nothing is known about the expression of the alternatively spliced transcripts for the α2(VI) collagen chain. We therefore set out to study the distribution of the two splice variants in several embryonic chicken tissues utilizing the technique of in situ hybridization in

combination with Northern blotting and the polymerase chain reaction (PCR).

MATERIALS AND METHODS

DNA Clones

Two DNA probes that recognize the two splice variants of the chicken α2(VI) collagen mRNA were used for Northern blotting and in situ hybridization experiments (Fig. 1). Clone 4aO contains cDNA sequences coding for part of the triple helix and for the adjacent carboxyl terminal VWFA repeat (nucleotides 1,296–2,500 of the cDNA) [Koller et al., 1989]. This sequence is present in both the major and the minor splice variant. A probe that specifically hybridizes to the minor splice variant was prepared from a genomic clone of the α2(VI) collagen gene. It represents a PstI fragment (PstB, 868 bp) that covers the entire translated sequence and part of the 3′ untranslated region of the alternatively spliced exon 28A (nucleotides 19,570–20,437 of the gene) [Hayman et al., 1991]. The two DNA probes were labeled with [α-^{32}P]dCTP or with [α-^{35}S]dCTP (Amersham, Buckinghampshire, UK) to a specific activity of approximately 10^8 cpm/μg by the random-primed oligolabelling method [Feinberg and Vogelstein, 1983].

Screening of cDNA Libraries

Probe PstB was used to screen three different cDNA libraries prepared in the cloning vector lambda gt11 (Clontech Laboratories, Palo Alto, CA) by the plaque-hybridization technique [Sambrook et al., 1989; Wälchli et al., 1993]. In each case approximately 5×10^5 recombinant phages were analyzed. One of the libraries had been produced from 10-day-old chicken embryos (CL 1001), one from adult chicken gizzard (CL 1011b), and the third from adult chicken lung (CL 1006b).

Northern Blots

Total RNA was prepared from various tissues of 17-day-old chicken embryos by the guanidinium thiocyanate method [Chomczynski and Sacchi, 1987]. Polyadenylated RNA was purified from total RNA by affinity chromatography on oligo(dT)-cellulose (Pharmacia LKB Biotechnology, Uppsala, Sweden). The mRNA (~ 2 μg) was resolved on a formaldehyde-containing 1% agarose gel, transferred to Nylon membranes and hybridized to the ^{32}P-labeled DNA probes under standard conditions [Sambrook et al., 1989]. After washing, the blots were exposed to X-ray film (Kodak X-Omat AR; Eastman Kodak Company, Rochester, NY) at −70°C using intensifying screens.

In Situ Hybridization

Tissue samples from 10- and 17-day-old chicken embryos were frozen in liquid nitrogen and cut into 5–8 μm sections. The cryosections were subjected to in situ hybridization under sterile conditions exactly as described [Wälchli et al., 1994]. They were fixed in paraformaldehyde, treated with proteinase K, and acetylated with acetic anhydride. Following dehydration in ethanol, the sections were prehybridized with salmon sperm DNA and then hybridized with the ^{35}S-labeled probes in the presence of 50% formamide. The slides were thoroughly washed, dried and dipped into nuclear track emulsion melted at 42°C. After exposure for 5–11 days in the dark they were developed. Before being mounted with Eukitt, the sections were counterstained with hematoxylin. Finally, they were examined and photographed with a Zeiss (Thornwood, NY) microscope equipped with dark-field optics [Wälchli et al., 1994].

Amplification by PCR

Three different oligonucleotide primers were purchased from Microsynth (Balgach, Switzerland) for the amplification of the major and the minor α2(VI) collagen transcript. The upstream primer Un (5′-TCTGCCCAGATCCACAGATTGTCTG-3′) was designed to recognize the major as well as the minor mRNA species. It hybridizes to a sequence coding for the second VWFA domain (nucleotides 2,528–2,552 of the cDNA) [Koller et al., 1989]. The downstream primer Ln (5′-GCGCATTCATGTTGTCATCGTTTCT-3′) recognizes specifically the major transcript. It hybridizes to a sequence that codes for the third VWFA domain of the α2(VI) collagen mRNA (nucleotides 2,716–2,740 of the cDNA). The other downstream primer La (5′-GCGTGGAGTTTAGGAACTGTGCGAT-3′) is specific for the minor transcript and encodes part of the motif with similarity to a fibronectin type III repeat (nucleotides 19,952–19,976 of the gene) [Hayman et al., 1991]. The three primers had the same length (25 nucleotides), a similar GC content and a similar optimal annealing temperature.

The downstream primers Ln and La (10 pmol of each) were annealed to poly(A)$^+$ RNA (2 μg)

by incubation at 65°C for 5 min in 10 μl of water. Reverse transcriptase from avian myeloblastosis virus (AMV, 10 u) was added and first strand synthesis was carried out in a buffer (final volume 30 μl) containing 60 mM Tris, HCl pH 8.3, 10 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM of each deoxynucleotide, and 1.8 u/μl of RNase inhibitor (all from Clontech Laboratories, Palo Alto, CA). After 30 min at 52°C the reaction was stopped by the addition of EDTA to a final concentration of 15 mM. Part of the template (0.5 μl) was subsequently amplified by Taq polymerase (1 u/sample; Life Technologies Inc., Gaithersburg, MD) in a thermocycler (35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C). The buffer contained 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, and 10 pmol of each primer (total volume 25 μl). After a final elongation step at 72°C for 10 min, the reaction mixture was extracted with an equal volume of chloroform and separated on a 8% polyacrylamide gel. The gel was stained with ethidium bromide and photographed under UV illumination (365 nm).

RESULTS

Occurrence of the Alternatively Spliced α2(VI) Collagen Transcripts

Previous studies had indicated the presence of a cryptic exon (termed 28A) within the last intron of the chicken α2(VI) collagen gene [Hayman et al., 1991]. Since the sequence of this exon possessed an open reading frame, a stop codon, a 3′ untranslated region, and a polyadenylation signal, it seemed likely that it was used in a mutually exclusive manner with the last exon of the gene (termed 28B) to generate two transcripts with different 3′ ends (Fig. 1). To gain information about the possible usage of the cryptic exon, we screened a cDNA library from chicken embryos with a genomic probe (PstB) covering the open reading frame of exon 28A. Unfortunately, all our efforts to identify a positive clone failed. Moreover, no positive clones were found in two additional libraries, one prepared from chicken gizzard, the other prepared from chicken lung.

We proceeded to search for the existence of the alternative transcript by Northern blotting experiments. Poly(A)$^+$ RNA was isolated from chicken embryos, separated on an agarose gel, and transferred to a Nylon membrane. Hybridization with probe 4aO, which spans a sequence common to both splice variants, revealed a broad band that migrated with a relative mobility corresponding to 4,200 nucleotides (Fig. 2A). Labeling of a similar blot with the genomic probe PstB which is specific for the alternatively spliced exon 28A produced a weak, but clearly visible band. This band migrated also with a mobility of 4,200 nucleotides as one would predict from the genomic DNA sequence. Since probe PstB does not recognize the major α2(VI) collagen transcript (sequence identity 38%), this result clearly demonstrates that at least two splice variants are transcribed from the chicken α2(VI) collagen gene. A rough estimation based on the intensity of the two bands suggested that the transcript, which included exon 28A, represented only a very small percentage of the total α2(VI) collagen mRNA. In the following, this transcript will be referred to as the minor splice variant in order to distinguish it from the major splice variant which includes exon 28B.

To see what tissues produce specifically one or the other isoform, we used the technique of in situ hybridization. In a first step we studied the expression of the major α2(VI) collagen transcript, since no systematic analysis about its distribution in chicken tissues was available. In a second step, we investigated the distribution of the minor transcript and compared it with that of the major transcript.

Tissue Distribution of the Major α2(VI) Collagen Transcript

In situ hybridization of cryosections from 6-day-old chicken embryos with cDNA probe 4aO showed a low and diffuse expression of the α2(VI) collagen mRNA. Neither the mesenchymal tissues of the limb rudiments nor the developing neural tube with all its associated organs (developing vertebrae, dorsal root ganglia, notochord, and perinotochordal matrix) revealed a distinct labeling pattern. A clear signal was only observed in the dermis and in the developing heart and gizzard. For this reason we confined our investigations to tissues from 17-day-old chicken embryos which show a differentiation state similar to that of adult tissues (Figs. 3–5).

On sections from 17-day-old embryos a bright signal was obtained with stromal fibroblasts from skeletal muscle. All the three layers of the muscular connective tissue (epimysium, perimysium, and endomysium) as well as the wall of small blood vessels were prominently labeled with our probe for the α2(VI) collagen subunit (Fig. 3A–D). Likewise, transcripts for collagen

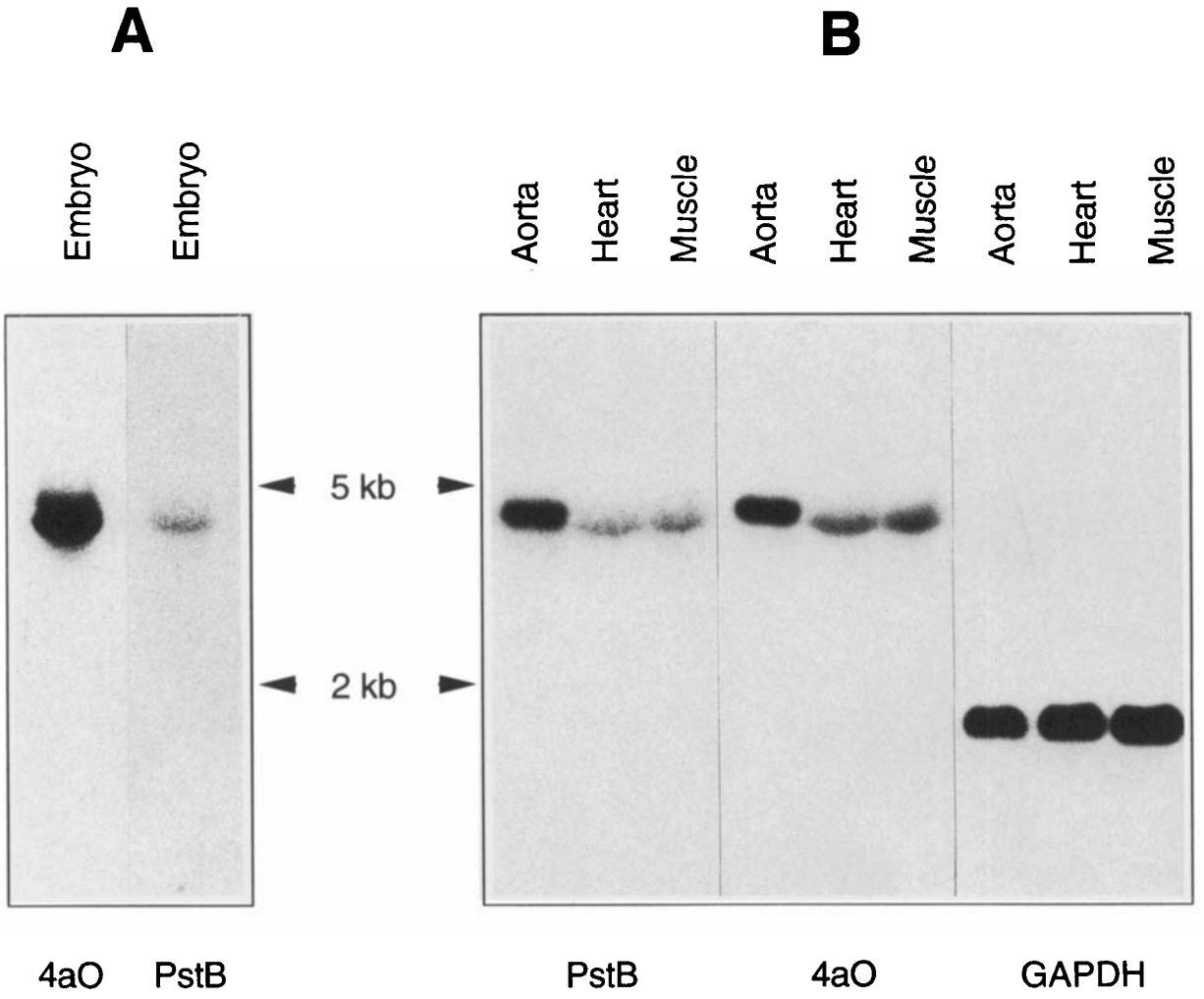

Fig. 2. Northern blot analysis. Poly(A)$^+$ RNA from entire chicken embryos (**A**) and from various chicken tissues (**B**) was resolved on an agarose gel, transferred to Nylon membranes, and hybridized to the radioactively labeled DNA probes 4aO (recognizing both transcripts), PstB (specific for the minor transcript), or GAPDH (specific for glyceraldehyde-3-phosphate dehydrogenase). The migration positions of the large and the small ribosomal RNA are indicated in the margin. Note that the blot hybridized to probe PstB was exposed for 4 days, while that hybridized to probe 4aO was exposed for only 4 h.

VI were detected throughout the stroma of other muscle types. In cardiac muscle, expression was abundant in the fine connective tissue septa (endomysium) and in the wall of the muscular arteries, but less prominent in the perimysium and the epimysium (Fig. 5A,B). In the gizzard, the dense fibrous sheets that separate the bundles of smooth muscle cells showed a strong reaction with our probe (Fig. 3E,F), as did the tissues associated with the gizzard muscular coat. The compact tendinous layer enclosing the whole gizzard as well as the submucosa on the inner side of the organ were also brightly labeled (Fig. 3I,J). Similarly, transcripts for α2(VI) collagen were detected in the smooth muscle layers of the intestine and in the invaginated lamina propria lining the intestinal wall (Fig. 3K,L). Collagen VI is therefore a major constituent of the interstitial septa of most, if not all muscular structures.

Connective tissues of parenchymal organs such as liver, kidney, and lung are also known to be rich in collagen VI. We have chosen chicken liver as a representative example. By in situ hybridization we found that transcripts for α2(VI) collagen were abundant in the portal stroma, but less prominent in the perisinusoidal space (Fig. 3G,H).

Next we investigated the situation in cartilage and bone since the presence of collagen VI in these tissues remains a matter of controversy. Our studies revealed a faint signal throughout

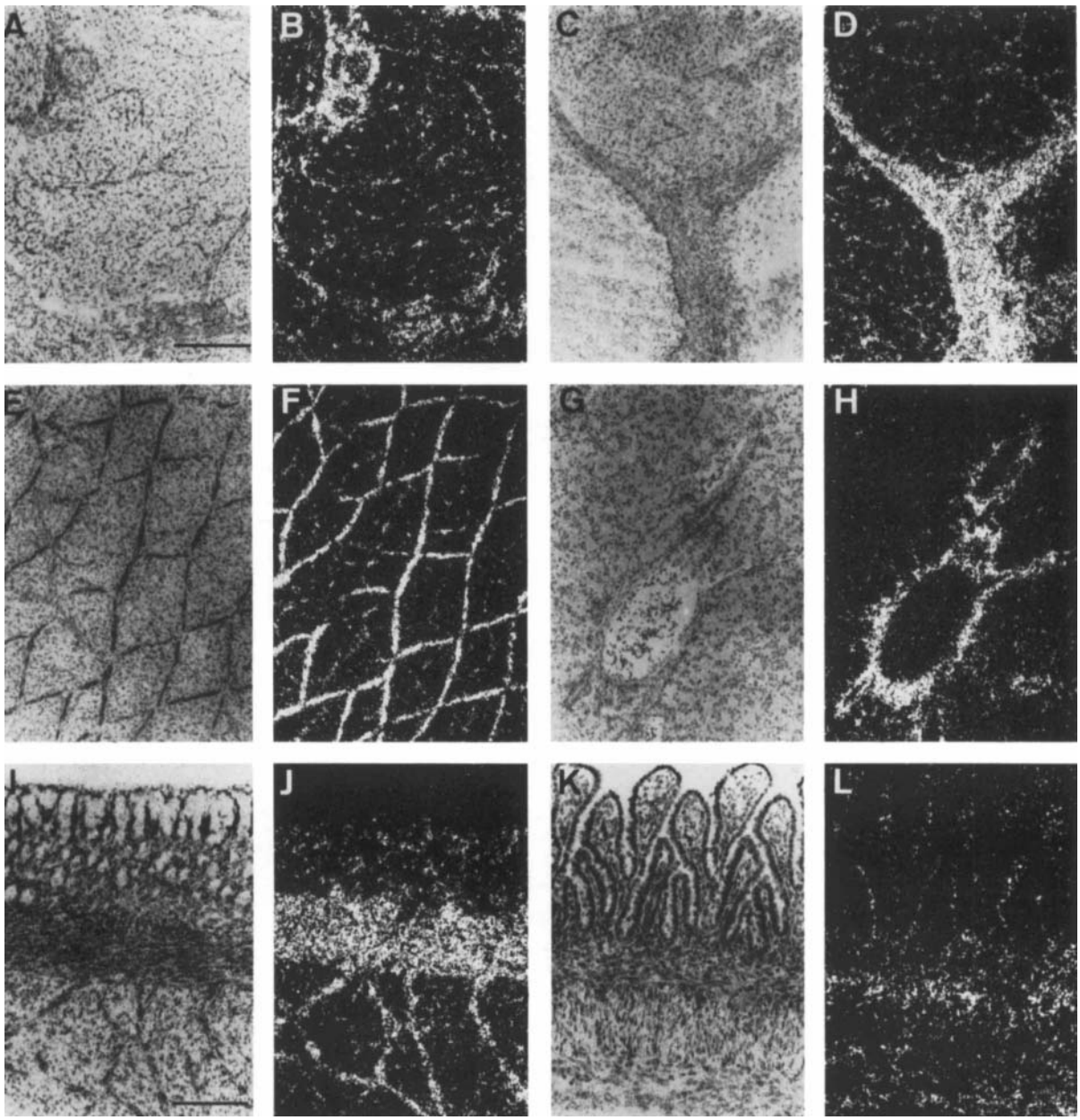

Fig. 3. Distribution of the major α2(VI) collagen transcript in tissues of 17-day-old chicken embryos by in situ hybridization. **A–D:** Skeletal muscle. **E,F:** Gizzard smooth muscle. **G,H:** Liver. **I,J:** Mucosa and submucosa of the gizzard. **K,L:** Wall of the small intestine. (A,C,E,G,I,K) brightfield; (B,D,F,H,J,L) darkfield. Bars = 200 μm (A–H); 100 μm (I–L).

the hyaline cartilage in developing ribs (Fig. 4A,B) and in epiphyses of long bones (Fig. 4C,D). This signal was barely stronger than the background observed in areas devoid of any tissue. In contrast, cells at the articular surfaces were prominently labeled with our α2(VI) collagen probe (Fig. 4C,D). Hyaline cartilage is characteristically covered by the perichondrium, a fibrous membrane that differentiates into the periosteum during the process of perichondral ossification. As shown in Figure 4A and B, high expression of collagen VI was observed in perichondrial cells around the cross-sectioned rib, particularly in the region where the perichondrium merges with tendons attached to the rib. At this stage of development, the sub-perichondral layers lie immediately adjacent to a thin collar of newly formed bone, which encloses the cartilage model.

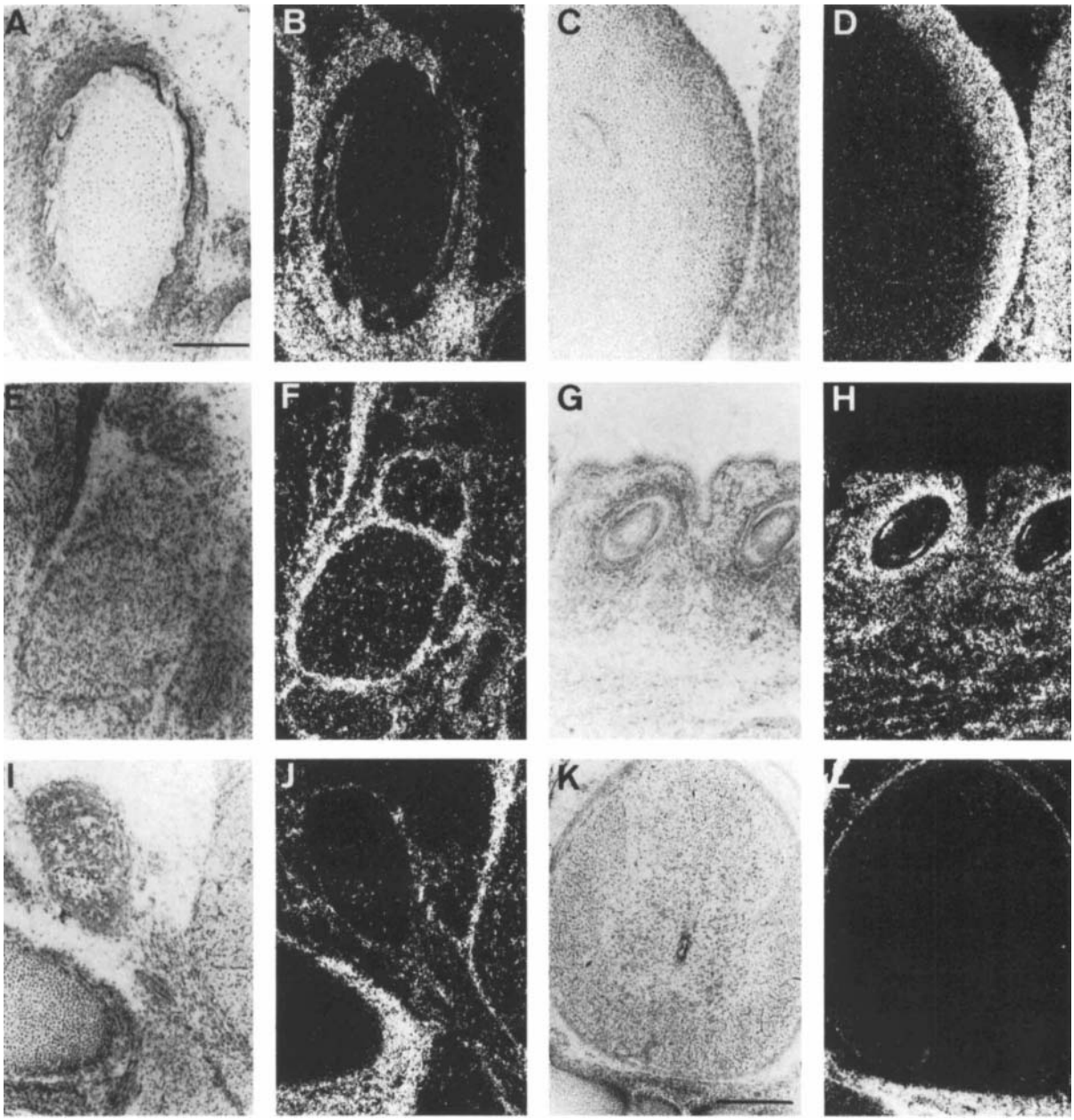

Fig. 4. Distribution of the major α2(VI) collagen transcript in tissues of 17-day-old chicken embryos by in situ hybridization. **A,B:** Cross-sectioned rib. **C,D:** Epiphysis of a long bone with articular cartilage. **E,F:** Peripheral nerves. **G,H:** Skin. **I,J:** Spinal ganglion. **K,L:** Neural tube. (A,C,E,G,I,K) brightfield; (B,D,F,H,J,L) darkfield. Bars = 200 μm (A–J); 400 μm (K,L).

These layers correspond to the internal part of the differentiating periosteum and contain osteoblast precursor cells. An irregular, patchy distribution of silver grains was detected in this structure, especially around blood vessels. Likewise, expression of collagen VI was found in the periosteal cells covering the completely ossified long bone (Fig. 5D,E). The bone matrix itself was also rich in grains, but the interpretation of this signal was difficult because of the unspecific light scattering effect of the osteoid under darkfield illumination. As seen at higher magnification, the silver grains were clearly accumulated over and around osteoblasts (not shown).

Several additional tissues with a high proportion of extracellular matrix were found to express significant amounts of collagen VI mRNA. In the skin, stromal fibroblasts throughout the

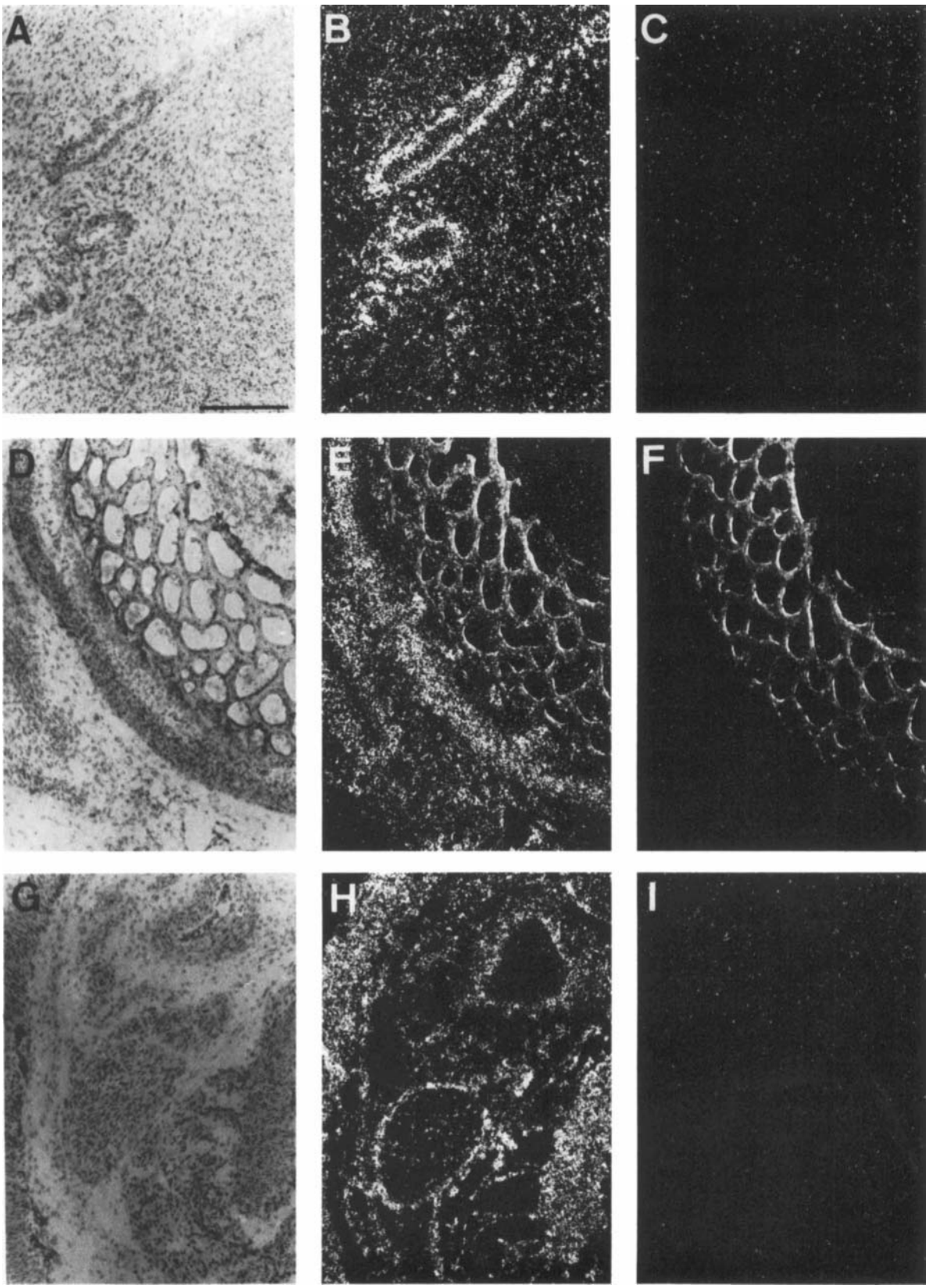

Fig. 5. Distribution of the major (A,B,D,E,G,H) and the minor (C,F,I) α2(VI) collagen transcript in tissues of 17-day-old chicken embryos by in situ hybridization. **A–C:** Cardiac muscle with blood vessels. **D–F:** Cross-sectioned femur. **G–I:** Skeletal muscle with nerves, blood vessels, and tendon. (A,D,G) brightfield; (B,C,E,F,H,I) darkfield. Bar = 200 μm.

dermis were labeled with the α2(VI) collagen probe, the signal being particularly strong around feather buds and capillaries (Fig. 4G,H). An intense signal was also observed in tendon (Fig. 3C,D), in the wall of muscular blood vessels (Fig. 5G,H), in the aorta (Fig. 6A,B,E,F), and in the epineurium and perineurium of peripheral nerves (Figs. 4E,F, 5G,H). The endoneurium associated with single axon/Schwann cell units was labeled in a punctate pattern. Finally, a signal was also observed in the spinal ganglia and in the emerging spinal nerve trunk (Fig. 4I,J). However, no expression of collagen VI was found in the neural tube. Only the thin layer of connective tissue cells around the neural tube was faintly labeled (Fig. 4K,L).

Distribution of the Minor α2(VI) Collagen Transcript

Every tissue described above was subsequently examined for the expression of the minor α2(VI) collagen transcript. Serial sections were hybridized with probe PstB specific for the alternatively spliced exon 28A or with probe 4aO used above and the labeling patterns were compared. Linearized pUC DNA served as a control probe and revealed a uniform distribution of silver grains, similar to the background observed in areas devoid of any tissue.

The majority of the tissues examined did not yield any specific signal with the probe for the minor transcript. Representative examples are shown in Figure 5. Cells in the vascular walls of cardiac (Fig. 5C) and skeletal muscle (Fig. 5I) that had been found above to express substantial amounts of the major splice variant did not reveal any labeling. The connective tissue septa (endo-, peri-, and epimysium) were also negative in both muscle types. Similarly, transcripts for the minor variant could not be detected in the periosteum and in the bone matrix (Fig. 5F). Only the mesh-like structure of newly formed bone appeared positive under dark-field illumination due to the unspecific light scattering effect. At higher magnification, however, there was no specific accumulation of silver grains over osteoblasts in contrast to the sections labeled with probe 4aO (Fig. 5E). Other tissues lacking any labeling with probe PstB included tendons and peripheral nerves (Fig. 5I). Thus, neither of the connective tissues shown above to be rich in collagen VI mRNA expressed the minor α2(VI) collagen transcript at a level detectable by in situ hybridization.

There was one remarkable exception. Cross-sections of the aorta revealed a clear signal in stromal cells throughout the aortic wall when hybridized with probe PstB (Fig. 6C,G). Although the labeling was weaker than that obtained with probe 4aO (Fig. 6B,F), it was significantly stronger than the background observed on control sections hybridized with pUC DNA (Fig. 6D,H). The media of the aorta is therefore the only tissue that produces the minor splice variant of the α2(VI) collagen mRNA at a detectable level.

Northern Blotting

The results obtained by in situ hybridization were now verified by Northern blotting experiments. Poly(A)$^+$ RNA was isolated from three chicken tissues, namely skeletal muscle, heart, and aorta. The RNA was separated on an agarose gel and transferred to a Nylon membrane. Hybridization with probe PstB specific for the alternatively spliced exon 28A followed by exposure of the blot for 4 days yielded a strong band with the RNA preparation from aorta (Fig. 2B). Substantially weaker signals were obtained with the RNA preparations from heart and muscle. The size of the band corresponded to 4,200 nucleotides consistent with the result obtained above (Fig. 2A). Aorta contains therefore a substantial amount of the minor transcript for α2(VI) collagen.

The blot was subsequently stripped and reprobed with clone 4aO which recognizes both splice variants. After a brief exposure of only 4 h, a very strong signal was observed with the RNA preparation from aorta (Fig. 2B). Considerably weaker signals were obtained with the RNAs from heart and muscle, but these signals showed up strongly when the autoradiogram was exposed for 4 days. Since the two probes PstB and 4aO had been labeled to a similar specific activity, we concluded that the major splice variant was at least twenty times (4 days as compared to 4 h of exposure) more abundant than the minor variant. It is important to note that the relative ratio between the minor and the major variant was fairly similar in the three mRNA preparations. Aorta contained the highest amount of the minor splice variant, but at the same time also the highest amount of the major variant. Since the proportion of the minor splice variant did not vary in the three tissues examined, it is obviously not expressed in a tissue specific fashion.

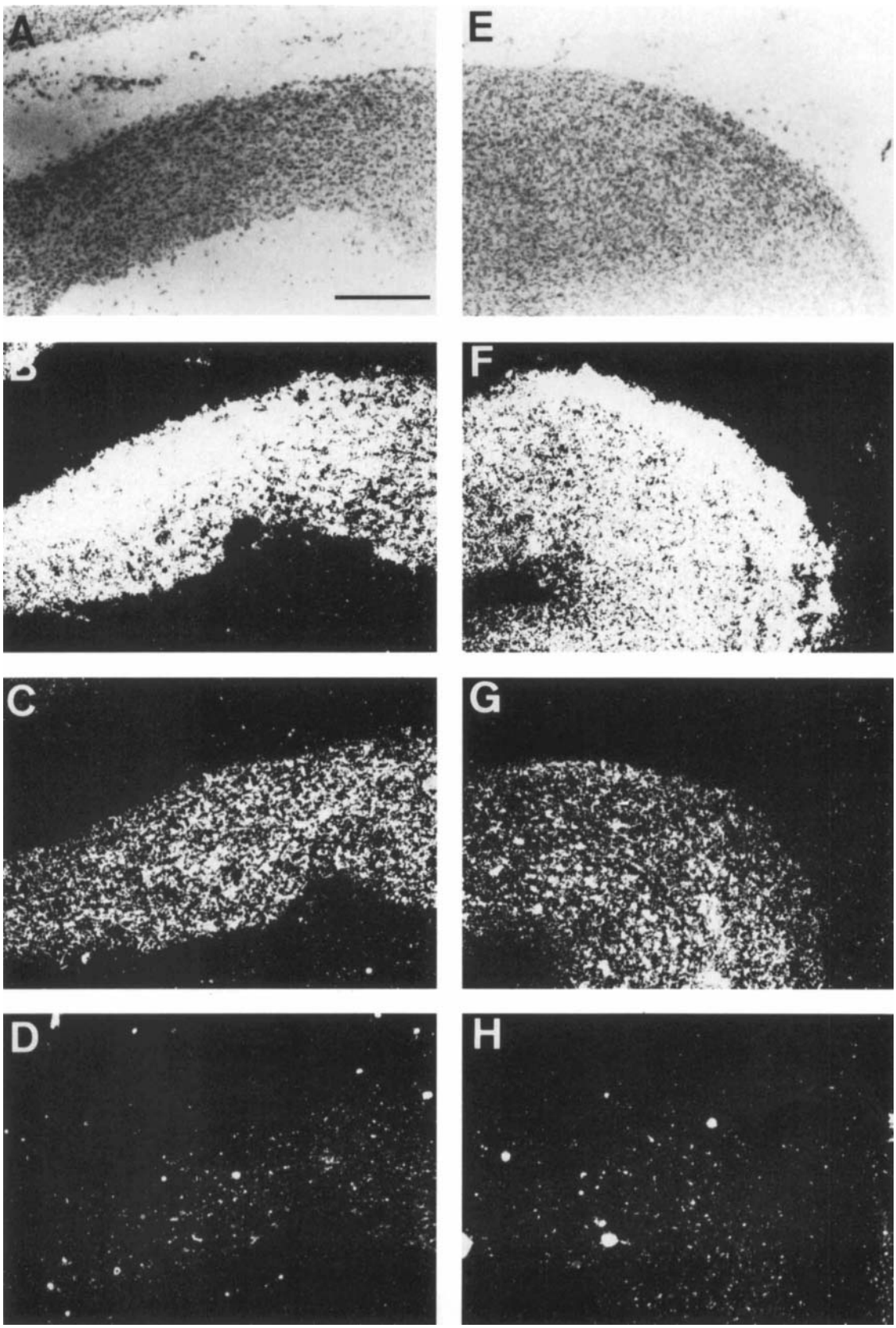

Fig. 6. Distribution of the major (**A,B,E,F**) and the minor (**C,G**) α2(VI) collagen transcript in the aortic wall of 17-day-old chicken embryos by in situ hybridization. (**D,H**) control hybridization with labeled pUC DNA. (A,E) brightfield; (B,C,D,F,G,H) darkfield. Bar = 200 μm.

To verify that the samples from aorta, heart, and muscle contained comparable amounts of RNA, the blot was hybridized to a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (a house-keeping enzyme). A strong band of 1,500 nucleotides was obtained with all three preparations, indicating that all samples applied to the gel contained a similar amount of total RNA (Fig. 2B).

Amplification by PCR

To confirm our conclusions we used PCR. Three oligonucleotide primers were designed, an upstream primer Un specific for the constant region of the two splice variants and two downstream primers Ln and La specific for the major and the minor α2(VI) collagen variant, respectively. With primer pair Un/Ln amplification of a 213 bp product (indicative of the major α2(VI) collagen transcript) is expected, whereas primer pair Un/La should yield a product of 162 bp (indicative of the minor transcript).

As demonstrated in Figure 7, products of the expected length were obtained after reverse transcription and amplification of the three mRNA preparations with the corresponding set of primers. When the template or either one of the primers was omitted, no distinct amplification product was observed proving the specificity of the reaction. When all three primers were added simultaneously, both products of 162 and 213 bp were obtained. The ratio between the lower and the upper band was found to be close to 1:20 in accordance with the result obtained above by Northern blotting. No differences were detected in this ratio when the samples from aorta, heart, and skeletal muscle were compared. Even when the templates were diluted tenfold before amplification, the ratio remained constant. The minor α2(VI) collagen variant is therefore expressed in a constitutive and not in a tissue specific fashion, at least in the tissues examined in this study.

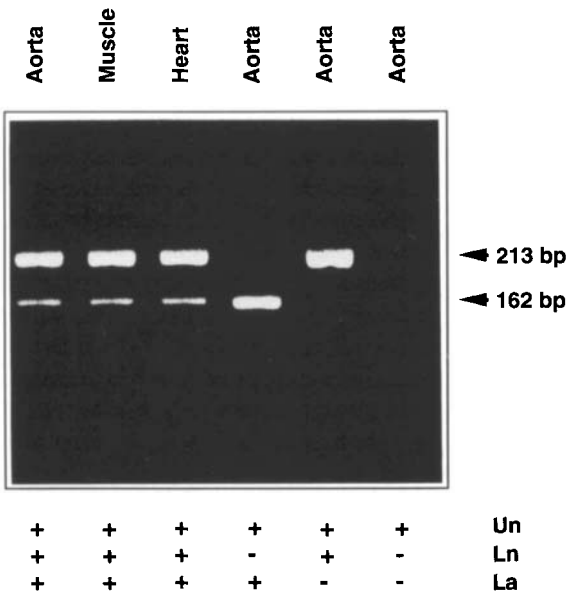

Fig. 7. Amplification of the major and the minor α2(VI) collagen transcript by PCR. Poly(A)$^+$ RNA from three different tissues was transcribed into cDNA by reverse transcriptase using primers Ln and La. Part of the cDNA was subsequently amplified by PCR with the primer sets indicated at the bottom of the panel. The migration positions of the 213 bp band (indicative of the major splice variant) and the 162 bp band (indicative of the minor splice variant) are shown in the right margin.

DISCUSSION

Collagen VI is a complex multidomain protein that mediates multiple interactions among connective tissue components. Several in situ hybridization studies have recently dealt with the expression of this collagen [Timpl and Chu, 1994, and references therein]. In situ hybridization allows the identification of a particular tissue—and even of the individual cell type within a tissue—that actively expresses the gene for a given protein. A distinction between the level of the mRNA and that of the final polypeptide product is of importance in the case of long-lived proteins including collagens, which gradually accumulate in the extracellular matrix. Such proteins remain detectable with antibody probes, even when the particular tissue has ceased transcription of the corresponding gene for some time. So far, nearly all studies reported in the literature used this technique to analyze differences in collagen VI expression under pathological conditions. The primary focus has been the human skin, where alterations in the level of the α2(VI) collagen mRNA have been demonstrated in cutaneous neurofibromas, systemic sclerosis, progressive diffuse fasciitis, keloids, and hypertrophic scars [Timpl and Chu, 1994; Peltonen et al., 1990, 1991; Sollberg et al., 1991; Unemori and Amento, 1991; Zhang et al., 1994]. In two other studies pulmonary fibrosis and peripheral nerves of diabetic patients were investigated [Specks et al., 1995; Muona et al., 1993]. There is only one report where the level of collagen VI mRNA was analyzed in a normal tissue, namely the human endometrium during the menstrual cycle [Mylona et al., 1995]. Our report represents therefore the first systematic analysis of

the distribution of the α2(VI) collagen mRNA in a variety of healthy embryonic tissues. This is also the first report that studies the situation in a nonmammalian system.

By in situ hybridization, a substantial amount of collagen VI transcripts was found in stromal cells of all tissues known to produce collagen I and fibronectin. The list includes tendon and skin, the septa of skeletal, smooth, and cardiac muscle, the portal stroma of the liver, the wall of blood vessels and the connective tissue of peripheral nerves. Our data confirm and extend earlier immunohistochemical studies performed by Gibson and Cleary [1983], Hessle and Engvall [1984], von der Mark et al. [1984] and Doliana et al. [1990]. Some controversy exists in the literature about the occurrence of collagen VI in cartilage and bone [Timpl and Chu, 1994]. We found that transcripts for this collagen were expressed primarily by chondrocytes near or at the articular surface, but at very low levels by chondrocytes in hyaline cartilage of developing ribs or long bones. Likewise, osteoblasts within the bone matrix contained only scarce amounts of α2(VI) collagen transcripts, while large amounts were seen in cells of the periosteum, both in the outer fibrous layer and in the inner osteoblastic layer. Thus, our results corroborate recent immunohistochemical data published by Keene et al. [1991] and Wardale and Duance [1993].

Alternative splicing of the primary gene transcript has been described for a variety of extracellular matrix proteins, including the collagens II, IV, XI, XII, XIII, XIV, and XVIII. Along with the usage of alternative promoters, this mechanism appears to play a significant role in the generation of additional diversity in the large collagen gene family [Boyd et al., 1993; Pihlajaniemi and Rehn, 1995]. In the case of collagen VI, alternative splicing has been demonstrated for the α2(VI) and the α3(VI) subunit, but not for the α1(VI) subunit. In both cases alternative splicing yields polypeptides that are lacking one or several VWFA domains. In the case of the α3(VI) collagen chain, no other module is incorporated into the final protein product in place of the skipped domain, but in the case of the α2(VI) collagen subunit the VWFA domain is replaced by a novel sequence motif. This motif possesses many characteristic amino acid residues typically conserved among fibronectin type III repeats. At the amino acid level, the motif shows 71% sequence homology between man and chicken. Since this motif has been conserved during evolution it is likely that it plays an important role which remains to be elucidated.

An idea about the function of this alternative splicing event can be gained by comparing the tissue distribution of the two splice variants. Indirect evidence for a differential distribution originates from cDNA cloning experiments. Clones for the major splice variant (α2C2, Fig. 1) were found in cDNA libraries from human placenta and human fibroblasts, but clones for the minor splice variant (α2C2a) were found exclusively in the library from placenta [Saitta et al., 1990]. Northern blotting experiments further demonstrated that the α2C2 variant was the predominant form in mRNA preparations from human fibroblasts, whereas the alternative transcript was hardly detectable in any preparation. These results are consistent with our findings obtained with the chicken transcripts. From PCR and Northern blotting experiments we estimate that the minor splice variant represents only a very small percentage of the total α2(VI) collagen mRNA. Why we have not been able to isolate any cDNA clone for the minor splice variant from a chicken cDNA library, although three independent libraries were screened, is not clear at the moment. Our in situ hybridization studies demonstrate that the major isoform predominates in all tissues examined. Although clearly detectable on Northern blots with poly(A)$^+$ RNA, the level of the minor isoform was too low to be detected by in situ hybridization.

One remarkable exception was noted. The wall of the aorta was found to contain a substantial amount of the minor isoform. At first sight this result suggested a tissue specific expression of the minor splice variant in aorta. Quantitative considerations, however, led us to conclude that the minor splice variant is expressed ubiquitously rather than in a tissue specific manner. PCR and Northern blotting studies showed that the minor transcript makes up less than 5% of the total α2(VI) collagen mRNA, even in aorta. Apparently, the wall of the aorta is labeled with the alternative probe since this tissue is extremely active in transcribing the α2(VI) collagen gene at the developmental stage investigated. The absolute amount of the minor splice variant therefore reaches a level that can be detected by in situ hybridization, although the

ratio between the two variants remains fairly constant in aorta, muscle, and heart.

If the isoform is ubiquitously expressed, it cannot serve a tissue specific function. It is therefore conceivable that it plays some role in the normal structure and assembly of collagen VI molecules. What comes to mind in this context is the network-like structure of the fine collagen VI microfibrils which exhibit many branching points. A small but constant percentage of the isoform could be required to generate branching of the microfibrils. If this were the case, the isoform should be locally concentrated at the branching points of the fibrils. This idea could now be tested by immunoelectron microscopy with antibody probes specific for the alternatively spliced products. In an attempt to tackle this question, we have raised polyclonal antibodies against the alternatively spliced domains of the chicken α2(VI) collagen chain produced as fusion proteins in an *Escherichia coli* expression system. Unfortunately, all antisera obtained had a very low titer and were not suitable for the experiment mentioned above (unpublished results).

More information is available about the expression of the alternatively spliced transcripts for the α3(VI) collagen chain. In contrast to the ubiquitously expressed isoform of the α2(VI) collagen subunit, at least one isoform of the α3(VI) collagen subunit exhibits a tissue specific expression. Doliana et al. [1990] were able to raise a monoclonal antibody against one of the alternatively spliced VWFA domains of this chain. With this antibody, the authors were able to demonstrate an accumulation of the corresponding isoform in the muscular layers of the intestine. In addition, tumor cells were found to synthesize an increased amount of this isoform when compared to normal cells, whereas fetal tissues produced a decreased amount [Stokes et al., 1991]. It would be of interest to see now whether the minor isoform of the α2(VI) collagen chain combines equally well with all isoforms of the α3(VI) collagen chain or whether a preferential combination of heterotrimers may exist.

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