Type X collagen is a recently discovered product of hypertrophic chondrocytes that is localized to presumptive mineralization zones of hyaline cartilage. Thus, in the epiphyseal growth plate of long bones it is present only in the zone of hypertrophic chondrocytes and absent in the resting and rapidly growing cartilage and in bone. Type X collagen represents, therefore, a transient and developmentally regulated collagen which is synthesized by a subpopulation of chondrocytes. We report here the isolation and characterization of cDNA and genomic clones specific for the chicken protein. The results demonstrate that the polypeptide chains of this collagen contain three distinct domains: a short non-collagenous, amino-terminal region, a collagenous domain of 460 amino acid residues, and a non-collagenous, carboxyl-terminal domain of 170 amino acid residues. The nucleotide sequence of the gene shows that these domains are encoded by a long open reading frame that is not interrupted by introns. The length and sequence characteristics of this segment raise the intriguing possibility that Type X collagen polypeptides may contain a transmembrane segment.

Collagenes are proteins that traditionally have been assigned a structural role in the extracellular matrix (ECM), considering the fibrillar structure of the macromolecular aggregates formed by the fibrillar collagens Type I, II, III, and V, it is indeed obvious that their major function is supportive and mechanical (for review see Ref. 1). However, fibrillar collagens have also been implicated in epithelial-mesenchymal interactions of importance for embryonic morphogenetic events (see Ref. 2). For example, epithelia when suspended in collagen gels can express characteristics of migrating mesenchymal cells (3), and soluble collagens added to the medium under a blebbing epithelial surface causes it to flatten (4).

Direct evidence for the mechanical role of Type I collagen comes from studies of the Mov13 mouse strain that carries a retroviral insertion in the α1(I) collagen gene (5, 6). The insertion causes a complete transcriptional block of the gene and results in an embryonic recessive lethal phenotype (7). Homozygous embryos develop normally up to about day 12 of gestation, but die at that time due to rupture of major blood vessels (8).

Although studies of the Mov13 mutant appear to rule out any major morphogenetic role of Type I collagen during the first 2 weeks of development in the mouse, the recent discovery of additional, genetically distinct, collagen types has raised the strong possibility that other collagens may have non-structural roles in ECM (9–16). A characteristic feature of many of these collagens (such as Types VI, IX, and X) is the presence of relatively large non-collagenous and short collagenous domains. Also, they do not appear to form ordered fibrillar structures similar to those of the fibrillar collagens Type I, II, III, and V. Many of these novel collagens represent minor components (in a quantitative sense) in various ECMS, and their molecular structure is different from that of the fibrillar collagens.

One group of novel collagens, Types IX and X, have been classified as short-chain collagens (17), because their biosynthetic molecules contain polypeptides that are shorter than Type I collagen polypeptide α chains. Type IX collagen has an unusual structure in that its molecules contain three triple-helical domains interspersed with non-collagenous domains (16). The molecules are disulfide-bonded and contain the polypeptide products of three different genes (18). We have recently isolated and characterized clones containing coding sequences from two of these genes, and we have shown that the Type IX collagen genes have an exon structure which is distinctively different from that of fibrillar collagen genes.

Type X collagen is a product of hypertrophic chondrocytes and has been localized to presumptive mineralization zones of hyaline cartilage (15, 19). Thus, in the epiphyseal growth plate of 12-day-old chick embryo tibiotarsus, Type X collagen is present in the cephalic half of the tissue, which will ossify, but absent from the caudal half, which remains cartilaginous. Type X collagen has also been detected as a product of chick embryonic sternal chondrocytes cultured in collagen gels with fibronectin-free medium (20) and in long-term cultures of hypertrophic chick embryo chondrocytes.

On the basis of these studies it appears that Type X collagen
represents a transient, developmentally regulated collagen which is synthesized by a subpopulation of chondrocytes. Previous studies indicate that, at least in the chicken, Type X collagen molecules are homotrimeric containing polypeptide subunits of 59 kDa (21). Limited pepsin digestion reduces the subunit molecular mass to 45 kDa (21). Electron microscopy of the two forms after rotary shadowing shows that the pepsin-resistant domain is a triple-helical, rod-like domain 132 nm in length (22). The intact 59-kDa form is similar in length but contains in addition a globular knob at one end. The chicken and rabbit molecules do not appear to contain inter-chain disulfide bonds (21, 23), but Type X collagen isolated from bovine cartilage has been reported to contain such bonds (24, 25).

To determine the complete primary structure of Type X collagen and to study regulation of the gene encoding this novel short-chain collagen, we have isolated and characterized cDNA and genomic clones specific for Type X collagen polypeptides. These studies demonstrate that Type X collagen contains a large non-collagenous domain at the carboxyl end of the molecule, and that the gene contains a long (about 2000 nucleotides) open reading frame without introns. Furthermore, examination of the amino acid sequence derived from the nucleotide sequence of the gene raises the interesting possibility that intact Type X collagen may contain a transmembrane segment.

MATERIALS AND METHODS

Cell Culture and Type X Collagen Isolation—Primary cultures of chondrocytes were prepared from zones 2 and 3 of the distal end of the tibiotarsus of 12-day-old chick embryos as described previously (21). The chondrocytes, isolated from a dozen embryos, were plated in 100-mm Falcon tissue culture dishes at a density of 100,000 cells/ml of medium. The cells reached confluence after a week of primary culture and were passaged into a secondary culture in 150-mm dishes. The secondary cultures reached confluence in a week, and we began the daily collection of culture medium. In the analyses described here, only material that had been synthesized in the presence of ascorbate, β-aminopropionitrile, and low serum was utilized.

The culture medium from all plates was pooled each day and made 5 mM in EDTA, 1.0 mM N-ethylmaleimide, and 0.3 mM in phenylmethylsulfonfyl fluoride. The pooled medium was clarified by centrifugation and ammonium sulfate was added to 30% of saturation. The precipitate was recovered the following day by centrifugation at 10,000 × g for 30 min, redissolved in a phosphate buffer at 4 °C containing the above protease inhibitors.

The collagen was further purified by a second ammonium sulfate precipitation as described above. The Type X collagen was separated from Type II collagen by a salt fractionation at acid pH. The collagen, solubilized in phosphate buffer, was dialyzed against 1.2 M NaCl in 0.5 M acetic acid. The Type II collagen precipitated under these conditions and was removed by centrifugation. The Type X collagen was subsequently collected by dialyzing the samples against two changes of 2.0 M NaCl in 0.5 M acetic acid, followed by centrifugation to recover the precipitate. All precipitates were redissolved in phosphate buffer containing protease inhibitors and stored frozen. The 45-kDa form of Type X collagen was prepared by limited digestion with pepsin at 4 °C for 16 h as described (21).

Purification of CNBr Peptides and Amino Acid Sequencing—The pepsin-resistant 45-kDa form of Type X collagen was digested with CNBr and the CNBr peptides were fractionated by high performance liquid chromatography as described (26). The instruments used were from Beckman and consisted of a Model 334 chromatograph, a Model 166 UV monitor, and a CRIB data system. The column was a Vydac TP 201 (4.6 × 250 mm) from the Separation Group, and it was protected with a guard column filled with pellicular C18 resin (Waters Associates). Elution was with a linear gradient of acetonitrile (12.8–46.8%, 10 min) in 0.1 M trifluoroacetic acid (26). Amino acid sequencing of selected CNBr peptides was performed using gas-phase sequencing from Applied Biosystems as described (18).

DNA and RNA Extracts—Total cellular DNA was extracted from red blood cells of adult chickens by homogenization, proteinase K digestion and phenol extraction. For construction of a genomic library from this DNA we used essentially the procedure described by Maniatis et al. (27). Partial and complete EcoRI digests of the DNA were combined, fractionated on sucrose gradients, and ligated to Charon 4A arms (New England Biolabs). A commercial packaging extract (Amersham Corp.) was used. Total cellular RNA was extracted from long-term cultured chondrocytes and 17-day-old chick embryo sterna using guanidinium hydrochloride as described (28). RNA was also extracted from the cephalic and the caudal half of 17-day-old chick embryonic sterna using this method. Poly(A)+ RNA was obtained by oligo(dT)-cellulose chromatography. The activity of the mRNA was assayed by cell-free translation using a commercial rabbit reticulocyte lysate. Translation products, labeled with [35S]methionine, were analyzed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate.

RESULTS

Cellular and Cell-free Synthesis of Type X Collagen Polypeptides—Chondrocytes liberated from tibiotarsus epiphyseal zones 2 and 3 of 12-day-old chick embryos were initiated for primary culture. Secondary cultures were maintained and the culture media harvested for up to 5 weeks (21). Type X collagen represents a major collagenous protein in these cultures, and it can be readily purified from other molecules as shown by SDS-polyacrylamide gel electrophoresis (Fig. 1). When samples are subjected to pepsin digestion before SDS-polyacrylamide gel electrophoresis (Fig. 1), the molecular weight of Type X collagen chains is reduced from 59 to 45 kDa (with collagen peptides used as molecular mass markers). As demonstrated previously (21, 22), this pepsin-resistant domain of the protein corresponds to the triple-helical, collagenous domain.

The presence of mRNA encoding Type X collagen polypeptides in extracts of cultured chondrocytes was assayed by cell-free translation. When poly(A)+ RNA, isolated from long-term cultures was used for translation, one of the major translation products (arrow in Fig. 2) could be identified as the α1(X) chain based on its sensitivity to bacterial collagenase and its molecular weight. As noted above, Type X collagen molecules appear to be homotrimeric of three identical polypeptides. The presence of a single band of translation product is consistent with this.

Isolation of a Pepsin-resistant Type X Collagen Fragment and Protein Sequencing—As demonstrated previously and illustrated above (Fig. 1), pepsin digestion of Type X collagen produces a resistant fragment containing three polypeptides each of 45 kDa molecular mass. The pepsin-resistant fragment was purified (21) and fragmented by cleaving at methionyl residues with CNBr. The CNBr peptides were separated by high performance liquid chromatography using a gradient of acetonitrile in heptfluorobutyric acid for elution (26). Two peptides were chosen for amino acid sequence analysis in the gas-phase amino acid sequencer. For each peptide 14 cycles
were run and the collected amino acid residues were identified. Fig. 3 shows the results of this analysis together with amino acid sequences predicted from the nucleotide sequence of the Type X collagen gene (see below). With the exception of the residue in cycle 10 of peptide 1, which could not be determined for technical reasons, and the post-translational hydroxylation of prolyl residues, the amino acid sequences determined by protein sequencing are identical to the sequences predicted from the nucleotide sequence.

Isolation and Characterization of a Type X cDNA Clone—
Based on the results of cell-free translation using RNA from long-term cultured chondrocytes (Fig. 2), we decided to use this RNA for construction of a cDNA library from which to isolate Type X collagen DNA sequences. For the initial screening of the cDNA library we used dot hybridization with labeled RNA probes. As shown in Fig. 4, one clone was strongly positive with 32P-labeled RNA isolated from long-term cultured chondrocytes (panel A in Fig. 4) but negative with RNA from 17-day-old chick embryo sterna (panel B). Since RNA from cultured chondrocytes is presumably enriched for Type X collagen mRNA, whereas 17-day-old chick embryo sterna are known (from immunofluorescence studies) to contain almost no Type X collagen (15), this cDNA clone, pYN3116, was characterized further by RNA transfer-blot analysis. As shown in Fig. 5, pYN3116 hybridized to RNA from the cephalic region of 19-day-old sterna and from long-term cultured chondrocytes, but not to RNA from the caudal region of 19-day-old sterna or whole 17-day-old sterna. These distributions of the RNA are consistent with the known distribution of Type X collagen as judged by immunofluorescence.

The size of the mRNA recognized by the insert of pYN3116

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**CELL-FREE TRANSLATION**

**RNA:** Long term cultured hypertrophic chondrocyte 17 day chick embryo sterna 19 day chick embryo sterna cephalic region

**Collagenase:** - + - + - +

**Fig. 2.** SDS-polyacrylamide gel electrophoretic analysis of cell-free translation products synthesized with RNA isolated from cultured chondrocytes and chick embryo sterna. The products were incubated with and without bacterial collagenase and electrophoresed in an 8% gel. Globular proteins were used as molecular weight standards. Note (arrow) the collagenase-sensitive band co-migrating with bovine serum albumin. As indicated in the text, we believe that this band represents the translation product of Type X collagen mRNA. Due to the anomalous migration of collagen polypeptides in SDS-polyacrylamide gels, the apparent molecular mass of this band is 69 kDa when compared with globular molecular mass markers, but only about 59 kDa when compared with collagen peptide molecular mass markers.

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**Fig. 1.** SDS-polyacrylamide gel electrophoresis of polypeptides recovered from the medium of tibiotarsal chondrocytes kept in culture for 4 weeks. Left lane, proteins obtained by precipitating the medium twice with 30% ammonium sulfate as described under “Materials and Methods.” The bands of α1(II) and α1(X) chains are indicated. Center lane, the 59-kDa form of Type X collagen isolated by acid salt fractionation of proteins recovered from the culture medium by precipitation with ammonium sulfate. Right lane, the 45-kDa form of Type X collagen prepared by digestion with pepsin. The size heterogeneity seen after pepsin is probably due to heterogeneities in pepsin cleavage sites at the amino or carboxyl ends of the 59 kDa molecules. The separation gel was 7% in polyacrylamide. Bands were visualized by staining with Coomassie Blue.

**Fig. 3.** Amino acid sequences determined for two cyanogen bromide peptides of Type X collagen, compared with sequences predicted from the nucleotide sequence of the gene (see below). For peptide 1 we were unable to determine the amino acid residue in cycle 10 from the sequencer for technical reasons.
DOT HYBRIDIZATION

Fig. 4. Analysis of cDNA clones by dot hybridization using $^{32}$P-labeled RNA from cultured chondrocytes (A) and chick embryo sterna (B) as probes. One clone, pYN3116, hybridized strongly to chondrocyte RNA but only weakly to sternal cartilage RNA. The positive clones in the lower part of both frames represent control cDNAs specific for Type II and Type IX collagens.

NORTHERN BLOT ANALYSIS

Fig. 5. Northern blot analysis of RNA obtained from the cephalic and caudal regions of 19-day-old chick sterna, long-term cultured chondrocytes, and 17-day-old sternal cartilage. Probes were nick-translated inserts from the plasmids pYN3116, pYN1738 (specific for $\alpha$1(X) collagen; 14), and pYN2142 (specific for Type II collagen; 43).
was 2500 nucleotides. The RNA could therefore accommodate the coding information for a 59-kDa polypeptide. Nucleotide sequencing of the pYN3116 insert demonstrated that although a typical collagenous sequence (Gly-X-Y), was not encoded by the cDNA, the sequence contained a 295-bp open reading frame starting at the 5′ end of the insert (see Fig. 7). Obviously, the cDNA did not represent a complete copy of the Type X collagen mRNA. Based on the size of the mRNA (2500 nucleotides) and the size of the cDNA insert (600 nucleotides) we estimated that the cDNA represented about 20% of the mRNA sequence.

Genomic Mapping and Isolation of the Type X Collagen Gene—Since the cDNA did not contain sequences encoding Gly-X-Y repeats while circumstantial evidence indicated that it probably was specific for Type X collagen, we decided to use the cDNA as a probe to screen for a genomic clone that would allow identification of collagenous sequences. To obtain such a clone we screened a chicken genomic EcoRI fragment library (see "Materials and Methods"), using the insert of pYN3116 as probe. A positive clone, YN92, containing a 16-kb insert, was isolated. By blot analysis we found that a 3.5-kb EcoRI fragment of YN92 hybridized to the cDNA (Fig. 6). This EcoRI fragment was therefore subcloned into the EcoRI site of pBR322 for further characterization (Fig. 6), generating the genomic subclone pYN92E1.

Hybridization as well partial sequence analysis demonstrated that the genomic clone YN92 contains the sequence of the cDNA pYN3116. In fact, with the exception of the poly A segment in the cDNA, the genomic clone contains the cDNA sequence without interruptions (Fig. 7). Within the region of overlap, the two sequences are identical, except for a single nucleotide at position 2260. The gene sequence contains an A at this position, whereas the cDNA contains a T. As a consequence a DdeI site is present in the gene but absent from the cDNA in this region (Fig. 7).

Sequence analysis of pYN92E1 also provided proof that the genomic clone YN92 and the cDNA pYN3116 encode the polypeptide chain of Type X collagen. As shown in Fig. 3, amino acid sequences of two CNBr peptides of Type X collagen are both encoded by pYN92E1. Furthermore, the sequence predicted from the nucleotide sequence of the genomic subclone contains (as expected) methionyl residues preceding each of the two peptide sequences.

To examine whether YN92 represents a unique chicken Type X collagen gene, chicken DNA was digested with several different restriction endonucleases and probed with a short PstI-PuulI fragment (180 bp) from the translated region of the cDNA pYN3116. The results of the blot analysis of these digests are shown in Fig. 8. With each of six different restriction endonucleases only a single band is observed. The size of this band (in kb) for each enzyme is the size predicted from the map of the genomic clone YN92 (Fig. 6). We conclude, therefore, that the genomic clone we have isolated represents the unique chicken Type X gene.

Nucleotide and Predicted Amino Acid Sequence of the Type X Collagen Gene—The complete nucleotide sequence of the insert of the genomic subclone YN92E1 contains an open reading frame extending from nucleotide 167 to nucleotide 2206. This open reading frame can be translated conceptually into the protein sequence shown in Fig. 7. Although the amino terminus of the protein cannot be clearly defined from this sequence, it is evident that a Gly-X-Y sequence, unique for collagenous polypeptides, starts at nucleotide 317 and continues to nucleotide 1696, followed by 170 amino acid residues of a non-collagenous sequence at the carboxyl end.

The Gly-X-Y sequence is 460 amino acid residues long, and the corresponding nucleotide sequence is not interrupted by introns. As demonstrated above, the sequence has been identified as the sequence of Type X collagen polypeptides. As to several imperfections in the triplet sequence structure, the number of residues in the collagenous domain is not a multiple of three. As shown in Fig. 7, the imperfections are of two types. One type of imperfection is a Gly-X sequence instead of a complete triplet Gly-X-Y; the second type of imperfection contains a Gly-X-Y-X-Y sequence in place of two triplets. Although the significance of these imperfections is unclear, it is interesting to note that out of five imperfections of the first type, three have the sequence Gly-Phe, whereas two contain the sequence Gly-Ile. Also of interest is that two out of three imperfections of the second type contain the sequence Gly-Leu or Gly-Ile, the sequence cleaved by mammalian collagenases within collagen triple helices. Examination of Type X collagen molecules by electron microscopy after incubation with mammalian collagenase in fact demonstrates that the enzyme cleaves the molecules in the two areas containing these Gly-Leu and Gly-Ile sequences (44). Considering the high level of synthesis of Type X collagen in mineralizing hypertrophic cartilage and the virtual absence of the collagen in bone tissue that replaces the cartilage (15), the degradation of Type X collagen by collagenases must be a relatively rapid process. This is supported by the finding that growth plates contain high levels of collagenase activity (33).

Examination of the conceptual translation product as presented in Fig. 7 reveals some striking and unusual distributions of specific amino acid residues in Type X collagen. As
FIG. 7. Nucleotide sequence of the Type X collagen gene and the amino acid sequence of the conceptual translation product. Non-collagenous sequences are indicated by boxed-in areas. Short horizontal arrows indicate amino acid residues that were confirmed by direct amino acid sequencing of two cyanogen bromide peptides of Type X collagen. As noted in the text, the collagenous domain contains two types of imperfections. These are underlined with single horizontal arrows for the short type and double lines for the longer type. The two non-collagenous sequences are indicated by Val Met Ala Asp Pro Lys Gly Leu Pro Gly Ser Gly Pro Arg Gly Gln Ser Gly.
noted previously from studies of the protein (21, 34), Type X collagen contains an unusually (for collagen) high number of methionyl residues in the triple-helical domain. This is confirmed by the gene sequence reported here. Out of 460 amino acid residues in the collagenous domain, 9 residues are methionyl residues (Fig. 9). A second striking feature is the concentration of tyrosyl residues in the non-collagenous, carboxyl-terminal domain. The nucleotide sequence predicts 13 tyrosyl residues in this 170-amino acid residue domain and only two tyrosyl residues in the rest of the molecule. This is in agreement with protein studies showing that most of a large number of tyrosyl residues were lost when the 59-kDa form of Type X collagen was digested with pepsin to generate the 45-kDa form (21).

The 170-amino acid residue carboxyl-terminal domain contains one potential attachment site for asparagine-linked oligosaccharide (Fig. 7). Whether this sequence, Asn-Gly-Ser, is utilized for carbohydrate attachment is not known.

We have not yet mapped the transcription start site in the Type X gene. Also, since our attempts to determine the amino-terminal amino acid sequence of the 59-kDa form of the protein have been unsuccessful, we cannot precisely define the start of translation. However, as shown in Fig. 7, the first methionyl codon (at nucleotide 191) after the start of the open reading frame at nucleotide 167 is followed by a lysyl residue and then a stretch of hydrophobic amino acid residues. It is possible that this sequence represents the signal peptide of the α1(X) chain. Assuming that this is the case, the amino-terminal, non-collagenous domain contains 42 amino acid residues (Fig. 9).

**DISCUSSION**

Type X Collagen is a Transient, Developmentally Regulated Collagen—Type X collagen represents the first example of a transient, developmentally regulated collagen in vertebrates. It is not synthesized by actively growing chondrocytes, and it is therefore not a component of most cartilaginous tissues. It appears to be a major product of hypertrophic chondrocytes, cells that are in the terminal stage of their maturation, and it is therefore present in the ECM of presumptive ossification zones of cartilage. This highly specific temporal and spatial expression of Type X collagen is clearly seen in the epiphyseal growth plates of long bones. The chondrocytes within this region pass through a maturation sequence that starts with small round cells of high mitotic activity (zone 1). The cells then go through a stage of flattened morphology with high rates of collagen and proteoglycan synthesis (zone 2) before they become hypertrophic in a zone of mineralized ECM (zone 3). Degradation of this mineralized matrix, invasion of vascular cells, and synthesis of bone ECM by osteoblasts finally lead to replacement of the cartilage by bone. Only cells in zone 3 appear to synthesize Type X collagen, and although immunofluorescence studies with a monoclonal antibody against the collagen have shown a faint fluorescence in bone, this signal is exceedingly faint as compared with the signal from hypertrophic cartilage (15). Also, attempts to isolate Type X collagen from calvarial bone have been negative.

Studies of Type X collagen synthesis by chick sternal chondrocytes provide a good illustration of the precise developmental regulation of the expression of this collagen. Prior to day 16 of incubation of fertilized eggs, Type X collagen cannot be detected in the sternum, whereas it is present as a major ECM component in the cephalic half of this tissue in 18-19-day-old embryos (19). Cells in the caudal half of 18-19-day-old embryonic sternum do not synthesize the collagen. The findings reported in the present study are consistent with these observations. Northern-blot analysis shows hybridization of the Type X cDNA to RNA from the cephalic half of 19-day-old sterna, but not to RNA from the caudal half of 19-day-old sterna or to RNA from whole 17-day-old sterna (Fig. 5).
The Type X Collagen Gene Contains a Long Open Reading Frame Encoding a Repetitive Collagenous Structure Without Intron Interruptions—Portions of genes encoding fibrillar collagen Types I, II, and III have been isolated from a variety of species. Characterization of the genes have demonstrated a remarkably high degree of similarity in their organization. The chicken pro-α2(1) gene was isolated first and it has served as a prototype for a fibrillar collagen gene (for discussion and further references, see Ref. 35). The gene spans 39 kb of DNA and contains 51 introns. 42 out of the 52 exons encode the collagenous domain of the pro-α2(1) chain. These exons range in size from 45 to 162 bp, with most being 54 or 108 bp in length. This finding has led to the hypothesis that the collagenous domain of fibrillar collagen genes evolved by multiple duplications of an ancestral gene containing a 54-bp coding unit (36). Duplication of the fully assembled gene in turn led to genes that encode the polypeptides of the various fibrillar collagens. The conservation of exon sizes among collagen Types I, II, and III genes may be a consequence of the need for conserving the precise molecular length and distribution of charged and hydrophobic amino acid residues along the polypeptide chains of these molecules, while maintaining the required Gly-X-Y repetitive structure (37).

Although Type IX collagen genes do not appear to conform to the exon structure of fibrillar collagen genes (38), multiple introns split the sequences coding for collagenous domains also in these genes. This finding supports the argument that a large number of introns in collagen genes may be a consequence of the need to prevent recombinational rearrangements due to their highly repetitive coding structure (see Ref. 39). An alternative explanation for the conservation of the large number of introns in different fibrillar collagen genes after they diverged from a common multi-exon precursor is that there was no selective advantage to favor genes that had one or more introns spliced out precisely (35). Whatever the reasons are for the conservation of a large number of introns in fibrillar collagen genes, it is surprising to find that the chicken Type X collagen gene contains a 1380-nucleotide-long open reading frame encoding a collagenous domain of 460 amino acid residues. The identification of the gene is based on the identity between the conceptual translation product of this open reading frame and amino-terminal amino acid sequences of two CNBr peptides derived from Type X collagen. These peptides are located close to the amino-terminal and carboxyl-terminal ends of the 460-amino acid residue collagenous domain. In addition, the predicted length of this domain is in excellent agreement with the number of amino acid residues calculated from the length (132 nm) determined for the triple-helical domain of Type X collagen by electron microscopy (22). In fact, if the value 0.286 nm is used for the length per residue along polypeptides in a triple-helical conformation (40), the triple-helical domain should be 462 amino acid residues as calculated from the electron microscopy data.

The Cloned Type X Collagen Gene Is Not a Pseudogene—Does the Type X collagen gene clone we have isolated represent a pseudogene, and is there a transcriptionally active Type X gene with multiple exons elsewhere in the genome? Several reasons lead us to conclude that the gene we have isolated is not an intron-less pseudogene. The first and most compelling reason is the result of the blot analysis shown in Fig. 8. When a 180-bp fragment of the cDNA is used as a probe, a single band is observed with chicken DNA digested with different restriction endonucleases. These bands correspond to the fragments obtained with the genomic clone pYN92. This clone must therefore represent the unique chicken Type X gene. The second reason is the sequence identity between the cDNA pYN9116 and the genomic clone pYN92E1. Except for the poly A segment of the cDNA and a single nucleotide in the untranslated portion at position 2280 (Fig. 7), 526 nucleotides of the cDNA are identical to the sequence of the genomic clone. The third reason is the sequence identity between the amino-terminal sequences of two CNBr peptides isolated from Type X collagen and the sequence predicted from the nucleotide sequence of the gene.

![Chicken Genomic DNA](image)

**Fig. 8.** Southern blot analysis of chicken genomic DNA cut with different restriction endonucleases and probed with a 180-bp fragment from the 5′ end of the cDNA pYN3116. Restriction endonucleases used were: EcoRI (E), HindIII (H), BamHI (B), PvuII (Pv), XbaI (Xb), BglII (Bg).

![Diagram](image)

**Fig. 9.** Diagram summarizing characteristic features of the Type X collagen translation product as deduced from the nucleotide sequence of the gene. Assuming that the methionyl codon at nucleotide 391 (see Fig. 7) represents the start of translation, the polypeptide contains 42 amino acid residues of non-collagenous sequence at the amino end and 170 residues of non-collagenous sequence at the carboxyl end. The collagenous domain is 460 amino acid residues long. The locations of short imperfections (Gly-X-Gly) within the collagenous domain are indicated by short horizontal bars; the locations of long imperfections (Gly-Y-X-Y-Gly) are indicated by squares. As noted in the text, the polypeptide contains a large number of methionyl residues (open circles) and a concentration of tyrosyl residues (filled circles) within the carboxyl-terminal, non-collagenous domain. The cysteinyl residues are indicated by open squares.
What are the consequences of the lack of introns in the Type X collagen gene? Because of the long, uninterrupted, and repetitive coding sequence of this gene one could argue that recombinational rearrangements occur more frequently in this gene than in fibrillar collagen genes. If this were true, one would expect a greater diversity among Type X collagens of different animal species than among fibrillar collagens. The reported existence of interchain disulfide bonds within the triple-helical domain of bovine Type X collagen (24) and the absence of such bonds in the chicken and rabbit (23) proteins, may reflect such a structural diversity. On the other hand, the conservation of length (45 kDa) of the pepsin-resistant triple-helical domain in Type X collagen from all three animal species suggests that major rearrangements have not occurred between birds and mammals. The answer to the above question is therefore not clear. A comparison of the Type X gene structure from other animal species with that of chicken should be helpful.

The homotrimeric structure of Type X collagen molecules may allow minor rearrangements of the gene without disruption of the triple-helical nature of their collagenous domain. In fact, as noted above, the amino acid sequence derived from the gene reveals several imperfections in the collagenous triplet structure (Fig. 7). Five imperfections are of the type -Gly-X-Gly- and three imperfections are of the type -Gly-X-Y-X-Y-Gly-. It is interesting to note that the same kind of imperfections are also present in α1(IX) and α2(IX) collagen chains. In Type IX collagen the imperfections must be compatible with the formation of triple-helical molecules from three different gene products, although this restriction does not apply to Type X collagen.

Does the Intact Form of Type X Collagen Contain Disulfide Bonds and a Transmembrane Segment?—Examination of the conceptual translation product of the Type X collagen gene reveals the presence of an intriguing hydrophilic sequence towards the carboxyl end of the non-collagenous domain. A hydrophilicity plot of the amino acid sequence of the non-collagenous, carboxy-terminal domain (data not shown) suggests the presence of a 29-amino acid residue sequence, between 2 lysyl residues, of mostly hydrophobic amino acids (Fig. 7). The sequence, whose nature and length is characteristic of polypeptides that span a membrane, contains 2 cysteinyl residues, 3 residues apart. Although electron microscopical data on the distribution of Type X collagen within the ECM of hypertrophic cartilage are not yet available, it is of interest to note that the matrix contains vesicles derived from the surface of chondrocytes (41). Therefore, an association between the membrane of matrix vesicles and Type X collagen is an intriguing possibility, even though immunofluorescence studies with a monoclonal antibody against this collagen show staining throughout the ECM (15). The 2 cysteinyl residues in the sequence are obvious candidates for residues forming interchain disulfide bonds in intact Type X collagen. However, such bonds have not been detected in the protein as isolated from the medium of cultured chicken chondrocytes (21). In addition, amino acid analysis of the medium protein did not show the presence of cysteine (21). It is possible, therefore, that the protein isolated from chondrocyte cultures represents a proteolytic cleavage product of Type X collagen from which the most carboxy-terminal segment (about 40 residues) has been removed.

In addition to the 2 cysteinyl residues within the 29-residue hydrophobic segment discussed above, the conceptual translation product of the gene (Fig. 7) contains a third cysteinyl residue in the middle of the carboxyl-terminal, non-collagenous domain and a fourth residue in the amino-terminal, non-collagenous domain. These two residues could well have escaped detection by amino acid analysis of the 59-kDa form of the protein (21), since no specific precautions were taken to ensure precise determination of cysteine during the analysis. In addition, since the gene sequence does not allow a precise definition of the amino terminus of the protein, we cannot at present exclude the possibility that the cysteinyl residue in the amino-terminal, non-collagenous domain of the conceptual translation product is not part of the secreted protein sequence.

Determination and Differentiation of Type X Collagen Expression in Chondrocytes—As discussed above, Type X collagen synthesis in chick embryonic sterna is only detected at about day 18 of incubation of fertilized eggs and only in the cephalic half of the cartilage. The appearance of the collagen is concomitant with the morphological hypertrophy of the chondrocytes in the cephalic region. When chondrocytes are isolated from the cephalic and caudal regions of more immature sterna (11–17-day-old embryos) and cultured with Type I collagen gels, cells from the cephalic region synthesize high levels of Type X collagen after 4 days in culture (42). The cells from the caudal region do not initiate Type X collagen synthesis when cultured under the same conditions. A possible explanation for this different response of chondrocytes taken from the two regions of the sternum is that the Type X collagen gene in cephalic cells is organized in a transcriptionally competent chromatin structure already at day 11, a week before it is actively transcribed in vivo, whereas the gene in caudal cells is in a stably repressed state. The availability of the cloned gene should permit analysis of this as well as alternative hypotheses.

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