To the Graduate Council:

I am submitting herewith a dissertation written by Weirong Shang entitled "Identification, molecular cloning, and developmental gene expression of a 34 kD protein produced by bovine extraembryonic membrane during early pregnancy." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate
School

IDENTIFICATION, MOLECULAR CLONING, AND DEVELOPMENTAL GENE EXPRESSION OF A 34 KD PROTEIN PRODUCED BY BOVINE EXTRAEMBRYONIC MEMBRANE DURING EARLY PREGNANCY

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Weirong Shang

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DEDICATION

This dissertation is dedicated to my beloved grandmother

Ms. Maine Z. Jin

and my parents

Mr. Hezhu H. Shang and Ms. Wenhua W. Zhu

and all the teachers in my life

Elementary school teacher, Ms. Wenying Gao

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ABSTRACT

Collagen is by far the most prevalent vertebrate protein as it constitutes approximately 30% of all proteins present in mammals. In fibrous or solid forms, the various collagens junction primarily as the principal supporting elements in a wide variety of connective tissues. Nevertheless, more recent researches strongly suggesting that collagen may play active roles in developmental processes, cell attachment, differentiation, growth and migration. In this dissertation, a major placental protein, designated $2B_9$, was identified as carboxyl-terminal propeptide of bovine collagen III during early embryonic development. cDNA clone of this protein was obtained and characterized. Developmental gene expression of this protein was studied in both bovine and ovine extraembryonic tissues. Results indicated that cDNA clone of $2B_9$ protein contained the entire 245 amino acid coding region for the carboxyl-terminal propeptide of bovine collagen III as well as the carboxyl telopeptide region and 3' untranslated region extension. On set of expression of bovine procollagen III mRNA was paralleled by the protein production, suggesting procollagen III regulation might be under transcriptional control. Coincidental expression of the procollagen III gene with development of bovine chorioallantois suggested an important role for collagen III in the early embryonic development. Bovine procollagen III gene contained multiple polyadenylation signals, differential usage of these signals

might be a characteristic of collagen genes during development.

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Background and Significance

1. Introduction

Early embryonic death has been described as the major cause of reproductive inefficiency in all classes of farm animals (Hansel, 1959). It has been estimated that 10% of all embryo fail to be fertilized (Gordon, 1976), 30% of all fertilized embryos die (Januideen and Hafex, 1980), and normally most of the embryonic mortality occurs prior to day 40 of pregancy. To reveal the reasons for these losses, abundant studies have been done to understand the process of early pregnancy. Numerous studies have demonstrated that interactions between the developing conceptus and maternal tissues are essential to the establishment and maintenance of pregnancy. However, much less is known about the role of the extracellular matrix during early pregnancy. It is believed that differentiation and behavior of cells during early embryonic development are not only controlled by humoral factors, but also by interactions with constituents of the surrounding extracellular matrix. The propose of this study was to identify an extracellular matrix protein, bovine α1 type III procollagen, and examine its role during bovine placenta development and early pregnancy. The specific objectives were:

1. Identification and molecular cloning of protein $2B_9$

produced by bovine extraembryonic membrane culture medium.

2. Developmental gene expression of $2B_9$ in bovine extraembryonic membranes during early pregnancy.

2. Early Embryonic Development in Cattle

After fertilization on day 1 in the oviduct, the embryo passes has developed to the morula stage and passes from the oviduct into the uterus by day 4 - 5. The fluid-filled blastocyst forms at about day 6 - 7 and is composed of an inner cell mass and a single layer of trophectoderm, which is also refered to as trophoblast (Betteridge et al., 1988). The blastocyst "hatches" from the zona pellucida on Day 9 - 10, expands and the inner cell mass pushes through the trophectoderm to form the embryonic disc (Betteridge et al., 1980). Over the next several days the blastocyst undergoes a remarkable growth and elongates to a filamentous structure beginning on day 14. By day 20 the blastocyst fills the gravid uterine horn and by day 24 extends into the contralateral horn (Bazer and First, 1983).

During blastocyst expansion, mesoderm cells migrate out between the trophectoderm and endoderm. The nonvascular mesoderm lines the trophectoderm to form the chorion, while vascular mesoderm overlies the endoderm forming the yolk sac (Greenstein et al., 1958), which will regress upon development of the allantois. On day 20, the allantois, composed of an

inner layer of endoderm and an outer layer of vascular mesoderm, migrates between the chorion and yolk sac. The allantois contains the plaental blood vessels and acts as a vascular bridge between the uterus and embryo. The amnion forms by cavitation surrounding the inner cell mass between days 13 - 16 (Greenstein, 1958). Between days 22 - 27, the allantois undergoes a dramatic expansion and progressively fuses with chorion until the chorioallantois is completely formed around the amnion between days 24 and 33 (Bazer and First, 1983).

In cattle, attachment of the fetal membranes to the endometrium is a gradual process that begins with loose attachment by interdigitating microvilli of the trophoblast and endometrial epithelium at about day 20 (King et al., 1982) and progresses to an attachment of the chorioallantois to the endometrial caruncles forming placentomes around days 30 - 36. Placentomes then become the major site of fetal maternal physiological exchange. Over the next 30 to 40 days the chorioallantois, cotyledon and caruncle form a interdigitating complex placenta (King et al., 1970).

3. Secretory Proteins of Extraembryonic Membranes during Early Pregnancy

From an evolutionary standpoint, adaptation of eutherian embryos to prolonged uterine gestation must have required development of self - sufficiency with respect to production

and secretion of complex biologically active molecules by the conceptus (Amoroso and Perry, 1975). In large domestic species including cattle, sheep and pigs, in which ectopic pregnancy does not occur, presence of conceptus tissues in utero prior to day 17 (Betteridge et al. 1978; Sreenan 1978; Northey and French, 1980), day 12 (Moor and Rouson, 1966a, b, c) and day 13 (Dhindsa and Dziuk, 1968; Bazer et al., 1982), respectively, results in prolonged maintenance of the corpus luteum. Luteostasis is only one of several conceptus - induced or mediated event associated with maternal recognition of pregnancy, others include establishment of immunologic priviledge and induction of histotrophe, which must occur if pregnancy is to be established and maintained (Cook and Hunter, 1978; Beer and Billingham, 1979). Clearly, therefore, conceptuses of these species must acquire the ability very early in development to produce and secrete biologically active molecules that effect a myriad of physiologic processes essential to maintenance of conceptuses throughout gestation.

Proteins synthesized and secreted by pre- and peri-attachment conceptuses of cattle (Bartol et al., 1985; Godkin et al., 1988), sheep (Godkin et al., 1982; 1984a; 1984b), ponies (McDowell, et al., 1990), and goats (Gnatek et al., 1989; Baumbach et al., 1990) have been characterized electrophoretically and a few proteins have been isolated and identified. Trophoblast protein-1 (TP-1) secreted from ruminant conceptuses has been identified as an interferon and

designated interferon tau (IFNτ). This protein is thought to initiate the maintenance of early pregnancy by binding to uterine receptors resulting in alteration of endometrial protein and prostaglandin metabolism (Reviewed by Roberts, R.M., et al., 1992; Bazer, 1992). Another low molecular weight protein has been identified as retinol-binding protein (RBP) (Liu et at., 1990; 1991; 1992; 1993). Retinol-binding protein is required for transporting vitamin A from the liver into the bloodstream where it travels to target tissues. RBP is expressed in the trophectoderm at the time of blastocyst expansion and maternal recognition of pregnancy (Liu et al., 1992). RBP has also been shown to be expressed by pig blastocysts and extraembryonic membranes (Trout et al., 1991; Harney et al., 1990; 1994). RBP may have a common function in regulating blastocyst development in mammalian species. Preimplantation bovine, ovine and porcine conceptuses also secrete a high molecular weight glycoprotein with a carbohydrate structure common to lactosaminoglycans (Masters et al., 1982; Newton and Hansen, 1988; Newton et al., 1988). This protein has been suggested to have immunosuppressive activity (Murray et al., 1987). In addition, bovine serum pregnancy-specific protein B (Butler et al., 1982; Sasser et al., 1986) and bovine pregnancy-associated glycoprotein (PAG) (Zoli et al., 1991) have been identified as members of the asparfic protease gene family. PAGs have also been identified as gene products of conceptuses of sheep, pigs and horses

(Green et al., 1994) detected and demonstrated to have roles in pregnancy.

Recently, another major placental protein, designated $2B_9$ (Godkin et al., 1988a) has been identified as bovine carboxyl-terminal propeptide of α1 type III collagen during day 24 - 45 of pregnancy (Shang, 1993). Collagens are originally synthesized in precursor procollagen forms, both amino and carboxyl terminal propeptides are removed extracellularly by specific propeptideases before the mature proteins assemble into microfibrils. Previous study which characterized protein production of bovine chorioallantois demonstrated that this extraembryonic membrane is active in synthesizing protein $2B_9$ (Godkin et al., 1988a). Since $2B_9$ is a member of collagen family members and is secreted during the critical time of rapid embryo and extraembryonic tissue growth and differentiation, the study of this protein may explore the important role of extracellular matrix during early embryo development and pregnancy.

CHAPTER 2

Literature Review

1. Introduction

The word collagen is a French neologism from the 19th century meant to designate the constituent of connective tissues that produces glue. The Oxford Dictionary (1983) defines collagen as "that constituent of connective tissue which yields gelatin on boiling". Many of the early studies were indeed done on heat-denatured collagen. On the other hand, the presence of fibers in connective tissues had been known since the 19th century from the work of early histologists Henle and Ranvier (van der Rest and Garrone, 1991). In the 1920s, Nageotte did pioneering work and determined that acid-solubilized collagen could precipitate into a material, later shown by X-ray diffraction and electroscopy to be collagen fibers (Eastoe, 1967).

Collagen fibers constitute the fundamental structural framework of all multicellular animals and has been estimated to account for about 25% of the body protein in mammals (Harkness, 1961). In more advanced animal species, collagen is prevalent in a number of disparate tissues and organs, suggesting that collagen molecules could originate biologically as products of a number of cell types. Most of the newly synthesized collagen molecules are utilized in the construction of extracellular matrix elements. The functional

role of collagen is achieved by these elements. But these elements demonstrate a great deal of morphological and architectural variability in different tissues and organs. For example, they are relatively massive striated fibers in tendons and ligaments, thin and often nonstriated in hyaline cartilages, and amorphous in a variety of thin membranous sheets such as basement membranes.

Studies of molecular heterogeneity of molecules comprising the matrix elements have provided information about the biological and architectural diversity of the collagenous elements. For example, genetically and structurally distinct collagens have been observed within the same organism (Miller and Matukas, 1969). According to genetically determined heterogeneity, it is now accepted that collagens collectively represent the products of a large multigene family. So far, at least 18 genetically and biochemically distinct types of collagen have been identified (Rehn and Pihlajaniemi, 1994). This family of proteins play a critical role in the development and maintenance of a variety of functions including tissue architecture, tissue strength, and cell-cell interactions (Miller, 1985).

2. Structure of the Collagen Molecule

Collagens comprise a large family of structurally related but genetically distinct proteins. Each collagen molecule contains three α chains associated with one another to form a

triple-helical conformation. This conformation is stabilized by interchain hydrogen bonds. These bonds are disrupted during thermal or chemical denaturation, resulting in unfolding of the molecule. Meanwhile, the peptide bonds linking adjacent amino acids are buried within the interior of the molecule, so that the triple-helical region is highly resistant to attack by promiscuous proteases such as pepsin. The only enzymes that efficiently attack the helical domain of native collagen molecules are collagenases (Linsenmayer, 1991).

Folding α chains into the proper helical conformation requires that glycine be present as every third amino acid residue. Therefore, the α chains are composed of a series of Gly-X-Y triplet sequences in which X or Y can be any amino acid. Usually, X is proline and Y is hydroxyproline. The presence of the imino acids proline and hydroxyproline is important for the stability of the triple helix. Hydroxyproline is formed by the posttranslational enzymatic conversion of proline in the Y position to hydroxyproline.

Like other secreted proteins, collagen molecules are originally synthesized in a precursor form, called procollagen. In procollagen molecules, each chain has large additional extension peptides, referred to as propeptides, at both NH_2 and COOH termini with molecular weights of 15,000 - 20,000 and 33,000 - 35,000 respectively. These propeptides are removed extracellularly from procollagen by specific procollagen N- and C- propeptideases. After removing of N- and

C-terminal propeptides, the collagen in the extracellular space only has short non-helical extensions (telopeptides) at both ends of triple-helical α chains (Jackson, 1980).

3. Cotranslational and Posttanslational Modifications of Collagen

Collagen is synthesized in the cell as single polypeptide chains in a precursor form carrying additional propeptides at both NH_2 and COOH termini. Along the biosynthetic pathway from the newly synthesized molecules to the fibril, the pro α chains undergo a series of posttranslational modifications, including hydroxylation of certain proline and lysine residues by specific hydroxylases, and N- and O-linked glycosylation by galactosyl and glucosyl transferases (Fessler and Fessler, 1978; Prockop et al., 1979). The complete triple-helical procollagen molecule is secreted into the extracellular space. The procollagen peptides are then enzymatically cleaved off, and the molecule thus acquires the capability to aggregate into fibrils.

A. Hydroxylation of Procollagen

Hydroxylations of prolyl and lysyl residues by two hydroxylases located in the rough endoplasmic reticulum (RER) are the best characterized modifications of procollagen (Prockop et al., 1979; Kivirikko and Myllyla, 1980; Adams and Frank, 1980). The two hydroxylases convert some prolyl

residues to 4-hydroxyproline and some lysyl residues to hydroxylysine. Nevertheless, the two hydroxylases require a peptide substrate in a nontriple-helical conformation, and the susceptible prolyl and lysyl residues must be within specific sequences (Kivirikko and Myllyla, 1980). Thus, prolyl-4-hydroxylase and lysyl hydroxylase will only hydroxylate prolyl and lysyl residues in the Y position of Gly-X-Y triplet sequences.

There is also a prolyl-3-hydroxylase which oxidizes proline residues in position X (Cardinale and Udenfriend, 1974). In the fiber-forming collagens, this has been observed only once in the α1(I) chain in the sequence Gly-3Hyp-4Hyp (Fietzek and Kühn, 1976). 3Hyp has been found more often in invertebrate than in vertebrate collagens.

B. Glycosylation of Procollagen

Following hydroxylation of lysyl residues, some of the hydroxylysine residues become glycosylated. The formation of O-β-D-galactosyl-hydroxylysyl and 1-O-α-D-glucosyl-O-β-D-galactosyl-hydroxylysyl residues is catalyzed by the two enzymes galactosyl transferase and glucosyl transferase. In addition to glycosylated hydroxylysyl residues, procollagen polypeptides also contain carbohydrate chains linked to asparagine (N-linked glycosylation) (Clark, 1979; Clark and Kefalides, 1976; 1978; Murphy et al., 1975; Pesciotta et al., 1981). This carbohydrate side chain is located in the

carboxyl-terminal half of the C-terminal propeptide in a region containing the sequence Asp-X-Thr/Ser, which is the structural requirement for N-linked glycosylation of asparaginyl residues by oligosaccharide transferases (Hart et al., 1979).

4. Nomenclature and Classification of Collagen Types

The development of the nomenclature system for the collagens and their constituent chains has been complicated by the continuous discovery of new type of collagen. The generally accepted system based on the traditional use of the lower case Greek letter α to designate single-chain components observed on denaturation of a collagen preparation. Additional letters such as β, γ and δ, have been reserved, respectively, to denote covalently linked dimers, trimers, and tetramers of the α chains. According to this system, the various chains identified as the primary constituents of collagen molecules have been mostly designated by the letter α plus an additional Arabic numeral notation that serves to distinguish each chain. The different type of chains, or different collagen types, are assigned by Roman numerals generally reflecting the chronological order in which they were purified and characterized. In some collagens, all three α chains are identical (e.g., collagen type II and III), in others all three α chains are different (e.g., collagen type VI), and in still others, two α chains are the same and one is different

(e.g., collagen type I). Thus, the type II and III collagens are designated $\alpha1(II)_3$ and $\alpha1(III)_3$, the type VI collagen with the three different chains is $\alpha1(VI)\alpha2(VI)\alpha3(VI)$, and the type I collagen with its two different chains is $\alpha1(I)_2\alpha2(I)$. The gene loci for the members of the collagen family have been given names beginning with COL, followed by an Arabic number denoting the collagen type, letter A (for A chain), and another Arabic number for the α chain in question. Thus COL1A2 is the gene locus for the $\alpha2(I)$ chain, and COL2A1 for the $\alpha1(II)$ chain.

5. Fibrillar Collagen - Molecular Organization, Gene Organization and Function

Based on their protein and gene structures, types I, II, III, V, and XI collagens have been assigned to fibril-forming collagen. Type I collagen is the major structural component in bone, skin, tendon, cornea and other fibrous tissue. Type II collagen is found in hyaline cartilage, the notochord, nucleous pulposus, and vitreous humor (Miller and Gay, 1987). Fibrils formed from type III collagen molecules are generally found in association with type I fibers in the more distensible connective tissues, such as, blood vessel walls, dermis, and placenta (Miller, 1971; Chung and Miller, 1974; Epstein, 1974). Aggregates derived from type V molecules occur in small fibrils in a number of tissues, bone, dermis, corneal stroma, and placental membranes, where they invariably

comprise only a minor fraction of the total collagen pool. Type XI collagen is thought to be the cartilage homologue of type V collagen, both in structure and in function (Eyre and Wu, 1987), and is found in hyaline cartilage and vitreous body (van der Rest and Garrone, 1991). This class of collagen molecules is derived through extracellular processing of large precursor (procollagen) molecules. All the procollagens studied thus far have the same overall structure as described in section 2, mainly referred to as triple-helical domain, N-propeptide domain and C-propeptide domain. Type I collagen, the prototype of the family, is a heterotrimer of two identical α1(I) chains and one α2(I) chain. Type II and type III collagens are homotrimers of α1(II) and α1(III) chains. the chain composition of type V collagen are variable, the most common structure is $\alpha1(V)_2\alpha2(V)$, but homotrimers of α1(V) have also been detected as well as heterotrimers α1(V)α2(V)α3(V) (Fessler and Fessler, 1987). The predominant form of type XI collagen is α1(XI)α2(XI)α3(XI) (Eyre and Wu, 1987).

A. The Triple-Helical Domain

The triple-helical domain comprises about 377 Gly-X-Y repeat triplets, any other amino acid sequence would disturb the triple-helical conformation. The triple-helical conformation is stabilized by the presence imino acids in the X and Y positions, the amino acids in these positions have

their side chains pointing outward from the helix, thus at the surface of the protein. This offers an exceptional potential for lateral interactions, particularly with other triple helices, which results in a higher stability or possible interaction with other extracellular matrix proteins.

The data of gene organization of this domain is based on the study of the available genomic sequences coding for the major fibrillar collagens, types I, II, and III (Sandell and Boyd, 1990). DNA sequence comparisons among these fibrillar collagen genes revealed that the triple-helical domain consists of 44 exons; 23 of those have a size of 54 bp, eight are 108 bp, and one is 162 bp, five exons are 45 bp and another five 99 bp long (Boedtker et al., 1985; de Wet et al., 1987; Upholt, 1989). The beginning and the end of the triple-helix are coded for by so called joining exons, which also code for telopeptide and propeptide sequences. Differences in the number of the Gly-X-Y triplets in the N- and C-terminal joining exons leads to the minor variations in the length of the triple-helical domain (1014-1029 amino acids). All of those Gly-X-Y coding exons are multiples of 9 bp (45, 54, 99, 108, and 162 bp coding for 5, 6, 11, 12, and 18 Gly-X-Y triplets, respectively). In all fibrillar collagen genes those triplets are uninterrupted. Since most of the exons are 54 bp long, it was suggested the ancestral gene for these collagen genes came from amplification of a DNA unit containing a 54-bp exon embedded in intron sequences (Yamada et al., 1980).

B. N-Propeptide Domain

At the N-terminal of all α chains of types I, II and III procollagen, there is a 23-amino-acid residue-long signal peptide which enables the nascent α chains to penetrate the membrane of the endoplasmic reticulum and is subsequently cleaved off (Kühn, 1987). The structure of the remaining N-terminal propeptide differs between α chains. In α1(I) and α1(III), they consist of following subdomains: a Cys-rich globular one of 66 and 68 residues in length stabilized by five disulfide bridges, a short triple-helical one of 39 residues [α1(III) chain] to 79 residues [α1(II) and α2(V) chains], and a short globular subdomain ending in the N-telopeptide. In the N-propeptide of α2(I), the Cys-rich globular subdomain is missing, so is the α1(II) chain. However, in N-terminal propeptide of α1(II) chain, its short triple-helical subdomain is 12 triplets longer than that of α1(III) chain (Kühn, 1987).

A variable number of exons (less than 10 exons, exact number depending on chain type) encodes the N-propeptide and the signal peptide. The Cys-rich subdomain is coded by exon 2 in the α1(I) and α1(III) gene but is not present in the α2(I) and α1(II). In the triple-helical subdomain, the triple-helical sequence contains one interruption in the α1(II) and two interruptions in the α2(V) chains. In the α2(I) and α1(III) genes this triple-helix is encoded by two exons, whereas the α1(I) chain contains an additional triple-helical

36-bp exon, and the α1(II) gene contains two 33-bp exons and one 54-bp exon (Vuorio and de Crombrugghe, 1990).

Two important functions are postulated for the globular domain of the N-terminal propeptide, (1) control of fiber formation in the extracellular space, which may also involve the C-terminal propeptide and will be described later, and (2) control of collagen biosynthesis. It has been observed that the Cys-rich globular subdomain of the N-propeptide specifically inhibits collagen biosynthesis of fibroblasts in culture and also in cell-free translation systems (Wiestner et al., 1979; Paglia et al., 1979). These observations led to the proposal that the N-terminal propeptide may be involved in a feedback control of types I and III collagen biosynthesis.

C. C-Propeptide Domain

According to the sequence of the cDNA, the C-terminal propeptides of all the four α chains [α1(I), α2(I), α1(II) and α1(III) are 243 to 247 residues in length. There is no triple-helical domain as is observed in the C-terminal propeptide. They contain eight cysteine residues, of which four are thought to be involved in intrachain disulfide bridges, which stabilize the globular structure. Another four [or three in α2(I), α2(V), and α1(XI)] cysteine residues form interchain bridges which are important for the stabilization of the complex of three α chains (Vuorio and de Crombrugghe, 1990). These interchain bridges are specifically formed at the

carboxyl-terminus of the C-propeptide and are a prerequisite for the formation of a triple-helical molecule (Uitto and Prockop, 1973).

The C-terminal propeptide regions of the α chains are strongly homologous to one another (Yamada et al., 1983a), the locations of the Cys residues and their neighboring sequences in the C-propeptides are strictly conserved as well as the sequence around the carbohydrate attachment site, suggesting the conservation of important function in these regions. The C-propeptide domain is coded by four exons (exons 49 to 52). The highest degree of sequence identity is seen in exon 51 around the carbohydrate attachment domain. This homology is also evident for the nucleotide sequence (Dion, 1987). The joining exon (exon 49) codes for the end of the triple-helix, the telopeptide, and the beginning of the C-propeptide. Another feature for fibrillar collagens is the presence of several polyadenylation sites in the gene transcripts. This gives rise typically to two mRNA species of approximately 5 kb and 6 kb (Myers et al., 1983; Aho et al., 1983). Variations in the ratio of the two transcripts have been detected, but the biological significance of these variations is not well understood (Penttinen et al., 1988).

There is some indirect evidence that the C-terminal propeptide is involved in alignment and selection of the α chains before triple-helix formation. Thus, the interchain disulfide bonds located in C-terminal propeptide of type I

collagen is formed before the triple-helix formation occurs (Uitto and Prockop, 1973), and it can be observed also if peptidyl hydroxylation and therefore triple-helix formation is inhibited (Fessler and fessler, 1974). The observation from embryonic chicken tendon fibroblasts synthesized in the presence of low concentration of puromycin shortened pro α chains. These generally lacked the C-terminal extension, and although relatively normal in hydroxylation, they were not triple-helical and were not secreted (Rosenbloom et al., 1976).

6. Transcriptional Regulation of Fibrillar Collagen Genes

Collective evidence indicates that the regulation of fibril-forming collagen synthesis by cells in culture or in developmental tissues occurs primarily by regulation of transcription rather than control of translation (Olsen, 1991). However, how the transcription of different fibrillar collagen genes is regulated is not well understood. The studies of type I, II, and III collagen genes suggest that the genes are regulated by very complex and diverse mechanisms (Ramirez and DiLiberto, 1990; Vuorio and de Crombrugghe, 1990). These mechanisms emphasize the precise tissue-specific transcription of the fibrillar procollagen genes during development and in adult organisms, and allow for modulation of their activity by growth factors, hormones, and cytokines.

As with other eukaryotic genes, fibrillar collagen genes

contain many important cis-acting regulatory elements (DNA sequences in and around the genes) located in a relatively small region of the 5' end to transcription start site. For example, when a 2000-bp 5' fragment from upstream of the transcription site of mouse α2(I) gene was placed next to the gene for bacterial chloramphenicol acetyl transferase (CAT), the CAT expression was high in tissues that expressed high levels of type I collagen and low in tissues that were low in type I (Khillan et al., 1986). These data indicate that sequences important for tissue-specific expression are located within the 2000-bp 5' upstream region in mouse α2(I) gene. Several specific sites for DNA-binding factors (trans-acting factor, regulatory proteins of cis-acting sequences) within this upstream promotor region have been identified in mouse α2(I) and α1(I) collagen genes. However, not all trans-acting transcription factors stimulate transcription. In mouse α2(I) gene there is evidence that two negative factors bind close to stimulatory binding protein (CCAAT-binding protein, Vuorio and de Crombrugghe, 1990).

7. Biological Function of Collagens

For a long time, only the biomechanical properties of collagen and its functions as a stabilizing scaffold of connective tissues for the organism were of research interest. In the 1960, it was realized that differentiation and behavior of cells during embryonic development are not only controlled

by humoral factors but also by interactions with constituents of the surrounding extracellular matrix (Hay, 1984).

A. Collagen in Cell-Cell Interaction

Collagen can interact directly with cells via specific receptors at the cell surface (Mollenhauer et al., 1983) or interaction can be mediated by other extracellular matrix components. According to cell attachment experiments, the first step of cell attachment is formation of a collagen fibronectin complex which then interacts via fibronectin with fibroblasts (Kleinman et al., 1979a). The best investigated work is the glycoprotein fibronectin which can serve as a mediator between collagen and the cell surface. Fibronectin has a particularly high affinity for denatured collagens (Jilek and Hormann, 1978). Using the peptides cleaved by cyanogen bromide from alpha chains of types I, II and III collagens, it was found that in all collagens the same region between residues 751 and 791 was responsible for fibronectin binding (Kleinman et al., 1976). This region overlaps with the cleavage site of vertebrate collagenase, evidence showed that the integrity of the collagenase - sensitive bound between residue 775 and 776 was required for fibronectin binding (Kleinman et al., 1979). This specific binding of fibronectin to collagen can be used to isolate fibronectin from tissue extracts or serum by chromatography on collagen affinity columns (Engvall and Ruoslahti, 1977). Fibronectin itself also

contains binding sites for collagen and for the cell surface receptors (Yamada, 1983; Yamada et al., 1985). Heparin appears to stabilize the binding of native collagen to fibronectin (Jilek et al., 1979). Rubin et al. (1977) suggested that hepatocytes do not require fibronectin for binding to collagen. Hepatocytes attach equally well to all fiber-forming collagens, whereas native collagen was more efficient in promoting cell attachment than denatured collagen. Since all cyanogen bromide peptides of type I collagen showed similar binding activities, it was suggested that cell binding sites in collagen have a fairly simple structure and occur in multiple copies along the α chains. This is in agreement with observations that collagen-like synthetic peptides also show a low but significant cell attachment effect (Rubin et al., 1984; 1986).

B. Collagen in Growth and Embryonic Development

Erhmann and Gey (1956) were the first to systematically compare growth of many cell strains and tissue explants on collagen substrates with growth on glass. They reported that collagen gels in many cases improved cell growth. Subsequently, it has been observed that collagen substrates alter the morphology (Elsdale and Bard, 1972), migration (Postlethwaite et al., 1976), and adhesion (Klebe, 1974; Hughes et al., 1979) of cells and, in some cases, differentiation (Meier et al., 1974).

Increasing evidence confirms that collagen plays critical roles in a number of morphogenetic processes, but the role which this class of molecules plays in embryo implantation processes is still uncertain. An early study showed that a collagen substrate promotes the differentiation of chicken embryo myoblast to myotubes (Hauschka and Konigsberg, 1966). In another early study in which crude collagen preparations were used, reconstituted collagen gels were found to be able to support embryo attachment and outgrowth (Jenkinson and Wilson, 1973). A more recent report indicates that six collagen types effectively supported embryo attachment and outgrowth. According to Carson et al. (1988), embryos acquired the ability to attach to collagens in two phases. Collagens II and VI were involved in earlier phase, embryo attached to collagen types I, III, IV, and V later than to the types II and VI during the implantation process.

Throughout gestation, trophoblast produces several hormones and "pregnancy" proteins (Bischof et al., 1984; Solomon, 1988; Talamantes and Ogren, 1988). Numerous in vitro studies have investigated different aspects of these hormones and proteins production. However, very few data are available concerning relationships between the extracellular matrix and hormone production by the trophoblast. Castellucci et al. (1990), showed that embedding trophoblast explants in three dimensional type I collagen gel and three dimensional fibrin gel is responsible for a much higher and longer lasting

production of hormones and trophoblast proteins compared to unembeded cultures. In mammary tissue, Hiroshi and Takami (1982) demonstrated that biosynthesis of type I and type III collagen is necessary for the hormone induction of functional differentiation of mammary epithelium in vitro.

Since collagen comprises a large heterogeneous class of protein molecules, of which at least 18 different types have been identified, various cells synthesize their own specific type of collagens and modify them to produce specialized structural matrices for the different cell types. Consequently, collagen expression is carefully regulated during differentiation and embryogenesis (von der Mark et al., 1976; Linsenmayer and Toole, 1977; Bornstein and Sage, 1980). More and more gene expression studies show that collagens have an extremely important role during embryonic development (Hay, 1991). Types I, III, IV, V, and VI collagens have been found in the utero-placental unit of humans, mice, and cattle (Sharp et al., 1990). 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 (Green et al., 1968). A Drosophila collagen gene has been shown to be expressed in a developmentally regulated manner (Monson et al., 1982). In the chicken embryo, collagens are produced in specific combinations in the growth and development of major connective tissues and in the developing organs (Romanoff, 1960; 1968).

C. Mutual Regulation between Transforming Growth Facter Beta Activity and Collagen Production

The remarkable growth and development of the early embryo and the extraembryonic membranes likely requires both endogenous growth factors and growth factors derived from uterine secretions. Extracellular matrix protein is thought to be involved in this process actively. The composition and organization of extracellular matrix is an important determinant of cellular behavior in that it regulates cellular adhesion, migration, proliferation, and differentiation (Ruoslahti and Pierschbacher, 1987; Ekblom, et al., 1986). It has recently been shown that growth factors play an important role in control of extracellular matrix. Of all the growth factors, none has been found to have the multi-effects on extracellular matrix ascribed to transforming growth factor beta (TGF β). This growth factor regulates not only the synthesis and degradation of specific extracellular matrix proteins, but also the ability of cells to respond to extracellular matrix via cell surface receptor, integrin (Roberts, et al., 1988; Wakefield, et al., 1987). Also, specific components of extracellular matrix, in turn, can both deliver TGF-β and regulate its activity (Roberts, et al., 1988; 1990; Roberts and Sporn, 1990).

The TGF-βs are disulfide-linked homo- and heterodimers of two related 12.5 kD peptide chains. Five distinct isoforms of

TGF-β have now been described, and three of these, TGF-β1, 2, and 3, are found in all mammalian species (Roberts and Sporn, 1990) with β1 and β2 being the most abundant forms(Cheifetz et al., 1988). Since receptors for TGF-βs are almost universally expressed (Wakefield, et al., 1987), effects of the TGF-βs have been demonstrated in a broad range of target cells, including hematopoietic cells, epithelial cells, and mesenchymal cells.

i. Effects of TGF-β on Synthesis of Collagen and Fibronectin

TGF-β stimulated synthesis and secretion of collagen and fibronectin was first described by Ignotz and Massagué (1986) in the culture of chicken embryo fibroblast cells. Since then this effect has been reported from several fibroblast cell lines of human, mouse, and rat origin (Roberts, et al., 1990). The range of extracellular matrix proteins known to be targets of TGF-β action has also been greatly increased, including types I, II, III, IV, and V collagen, and fibronectin (Roberts, et al., 1992). TGF-β1 and β2 have been found to be equipotent in the induction of matrix protein synthesis in every instance where it has been studied. Moreover, it has been found that TGF-β increases mRNA levels for most of the matrix proteins in which this has been examined and results in increased secretion of the protein. Specifically, it has been

shown that TGF-β can stimulate transcription of the mouse α2(I) collagen gene directly through a binding site for the transcription factor, nuclear factor 1 (NF-1), located in the promotor region of that gene (Rossi, et al., 1988). This same binding site accounts for part of the stimulatory action of TGF-β on transcription of the fibronectin gene (Dean, et al., 1988; 1989). It has more recently reported that TGF-β stimulated transcription of the human α2(I) collagen gene promoter by increasing the affinity of a different cis-acting element that contains a binding site for specificity protein 1 (Sp1) (Inagaki, et al., 1994). In addition, TGF-β has also been shown to stabilize both collagen and fibronectin mRNAs (Penttinen, et al., 1988; Raghow, et al., 1987). *In vivo* study of the expression of TGF-β and type I and III collagens, fibronectin, and proteoglycan during morphogenesis in the developing mouse lung demonstrated co-localization of TGF-β1 and these matrix proteins in mesenchymal cells surrounding bronchiolar and alveolar ducts and in clefts of branching ducts (Heine, et al., 1990). This data provided strong in vivo evidence supporting data from in vitro studies of the role of TGF-β in regulation of extracellular matrix formation.

ii. Effects of TGF-β on Degradation of Extracellular Matrix

The direct effects of TGF-β on synthesis of extracellular matrix proteins are enhanced by its ability to interfere with proteolytic degradation of newly synthesized matrix proteins. This occurs at two levels: (1) TGF-β decreases synthesis and secretion of several different proteases that act on extracellular matrix proteins and (2) increases synthesis of specific inhibitors of those proteases. For example, TGF-β reduces secretion of two common proteases, collagenase and plasminogen activator, and it increases synthesis of tisssue inhibitors of metalloproteinases and plasminogen activator (Edwards, et al., 1987; Overall, et al., 1989). Also, the effects of TGF-β on synthesis of both of these classes of the enzymes can be seen at the gene transcription of their respective mRNAs.

iii. Effects of TGF-β on Expression of Integrin Receptors

Integrins are specific cell-surface adhesion protein receptors. These receptors constitute a family of glycoproteins that both bind extracellular matrix components to the cell membrane and also link with cytoskeletal elements on the cytoplasmic side. The integrin receptors are noncovalent heterodinmers and consist of an α and β subunit. Three distinct families of integrin receptors are defined by

the type of β subunit; within each class, binding specificity is a function of the specific α subunit of the $\alpha\beta$ dimer (Hynes, 1987). Ignotz and Massagué (1987) have also described another important action of TGF-β in controlling the interaction of cells with extracellular matrix proteins. In their studies, they demonstrated that TGF-β increases both the level of integrin receptor mRNA and the rate of the receptor subunit processing by cells. The subunits of the $\alpha\beta1$ and $\alpha\beta3$ integrins, which mediate binding of cells to collagen, fibronectin, and vitronectin are all upregulated by TGF-$\beta1$ and $\beta2$ (Ignotz and Massagué, 1987; Heino, et al., 1989). These results suggest that TGF-β might control cell migration and differentiation not only through regulation of the composition of extracellular matrix, but also by specifically modulating the ability of the cell to adhere to different components of the extracellular matrix (Heino and Massagué, 1989).

iv. Role of Extracellular Matrix in Modulating the Effects of TGF-β

Although many of the studies of TGF-β on growth and differentiation of cells in culture can be related to its effects on extracellular matrix, extracellular matrix proteins could also regulate the function of TGF-β. It has been found that TGF-β activities can be inactivated by binding to several

matrix proteins such as type IV collagen, two proteoglycans, betaglycan , and decorin (Paralkar, et al., 1991; Andres, et al., 1989; Yamaguchi, et al., 1990). Synthetic peptide Arg-Gly-Asp blocked the binding of fibronectin to its receptor and interfered with the ability of TGF-β to stimulate growth of NRK (normal rat kidney) cells in soft agar (Ignotz and Massagué, 1986), suggesting that the increased secretion of fibronectin resulting from TGF-β treatment of these cells plays a role in the acquisition of anchorage-independent growth by the cells. In another system, it has been shown that the growth inhibitory effects of TGF-β on endothelial cells are probably mediated by its ability to increase cellular fibronectin secretion (Madri, et al., 1988).

8. Collagen Involvement in Connective Tissue Disorders

As described above, collagens are the most abundant of mammalian proteins and are distributed throughout the body. They form a scaffold on which structure of tissues is built, providing strength and integrity. Collagen serves primarily as a structural component in organs that bear weight, transmit force or light, protect or compartmentalize, and distribute fluids. Forming an important constituent of basement membranes, they can affect body hemostasis through interaction with platelets. Further more, they determine whether processes such as metamorphosis, organogenesis, tissue remodeling and wound healing will follow a normal or pathological course.

They are also essential for the epithelial-epithelial and epithelial-mesenchymal interactions and play important roles in cell differentiation.

While the growth and differentiation promoting effects of collagen is associated with its precisely timed synthesis, the production of abnormal collagen type results in alteration in tissue formation and metabolism. Thus, not only is collagen the major structural protein which functions via its fibers to stabilize the body organs, but it is also essential for cell-cell interaction and cell differentiation. Therefore, all alterations in its structure or metabolism may further affect cell function. In humans, a variety of connective tissue disorders are caused by defective collagen biosynthesis. For more than 10 years, studies have been focused on testing the relatively simple hypothesis that the degeneration of conncetive tissue characteristic of many diseases is the result of genetic defects in the collagen. A number of results have demonstrated that over 90 percent of patients with osteogenesis imperfecta (brittle-bone disease) have mutations in either the gene for the pro α1(I) chain or the gene for the pro α2(I) chain of type I procollagen (Prockop, 1992). More than 79 different mutations in these two structural genes have been identified in unrelated patients with osteogenesis imperfecta (Byers, 1990; Kuivaniemi, et al., 1991). Some of the mutations cause decreased synthesis of pro α1(I) or pro

α2(I) chains, but most cause the synthesis of structurally abnormal but partially functional chains. The disadvantage of the mutations causing osteogenesis imperfecta indicates that each of the repeating tripeptide sequences of G-X-Y that make up the about 1000- amino acid triple-helical domain must have the correct structure. A single mutation that makes one chain shorter or longer or that replaces a glycine residue with an amino acid that has a bulkier side chain can completely prevent the folding of the three chains and cause their degradation, in a process referred to as "procollagen suicide" (Prockop, 1984; 1990; Kuivaniemi, 1991). Moreover, every subunit of collagen that participates in the formation of fibrils must have the correct structure. In osteogenisis imperfecta, some of the mutations that substitute amino acids with bulkier side chains for glycine residues do not interfere with folding of the protein into a triple helix. Instead, they produce subtle changes in the conformation of the protein that alter fibril formation (Vogel, et al., 1988).

Mutations in type II collagen is a cause of chondrodysplasia, a highly heterogeneous group of heritable disorders of cartilage characterized by dwarfism, joint deformities, and other skeletal anomalies (Spranger, 1976). It has recently discovered that at least four mutations in the gene for type II procollagen in patients with lethal chondrodysplasias. Like the mutations that cause osteogenesis imperfecta, they are deletions, insertions, or glycine

substitutions (Kuivaniemi et al., 1991).

Mutations in type III collagen cause lethal Ehlers-Danlos syndrome, characterized by a laxity of joints, usually associated with skin changes such as hyperelasticity, thinness, and abnormal scarring (Byers, 1983). At least nine types of Ehlers-Danlos syndrome have been defined clinically. The most severe form is known as type IV and is characterized by the sudden rupture of large arteries or intestines. More than ten mutations in the gene for type III procollagen have now been found in probands with type IV Ehlers-Danlos syndrome (Kuivaniemi, et al., 1991). As might be expected, the mutations are similar to the mutations in type I procollagen that cause osteogenesis imperfecta, in that some cause the substitution of amino acids with bulkier side chains for glycine residues at specific positions along the pro α1(III) chain of type III procollagen. Other mutations cause the synthesis of shortened pro α1(III) chains because of defects in RNA splicing or partial gene deletions (Kuivaniemi, et al., 1991).

Although it is now evident that the hypothesis researchers have been testing applies to some common diseases in which tissues fall apart, it is not yet clear what fraction of the millions of people with the diseases have mutations in collagen genes. Further studies will contribute to provideing answers to this question and may lead, some day, to therapeutic regulation of collagen metabolism in tissues.

9. Conclusion

The collagen gene family comprises a large number of genes whose products constitute the major protein scaffold components of the extracellular matrix. Although we do not yet know the precise function of all those proteins, it is already evident that they play important roles in embryonic development and tissue morphogenesis. Obviously, it is not possible to appreciate how cells cooperate to build tissues without understanding how the structural molecules of the cells and their environment guide, constrain, facilitate, and generate cell behavior. This present study should contribute to our understanding of the molecular basis for collagen production during development and morphogenesis.

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CHAPTER III

Identification and molecular cloning of a 34 kD protein produced from bovine extraembryonic membranes

1. Introduction

Identifying proteins produced by the embryo and extraembryonic membranes during early pregnancy is a very important step which contributes to further understanding the mechanism of establishment and maintenance of pregnancy and the process of embryo development. Proteins synthesized and secreted by pre- and peri-attachment conceptuses of cattle (Bartol et al., 1985; Godkin et al., 1988), sheep (Godkin et al., 1982; 1984a; 1984b) and goats (Gnatek et al., 1989; Baumbach et al., 1990) have been characterized electrophoretically and a few proteins have been isolated and identified. Trophoblast protein-1 (TP-1) secreted from ruminant conceptuses has been identified as an interferon and designated as interferon tau (IFNτ). This protein is thought to initiate the maintenance of early pregnancy by binding to uterine receptors resulting in alteration of endometrial protein and prostaglandin metabolism (Reviewed by Roberts et al., 1992; Bazer, 1992). As production of INFτ diminished between days 24 - 29 in the cow and 21 - 24 in the ewe, a low molecular weight protein has been identified as retinol-binding protein (RBP), which is required for transporting vitamin A from liver into the bloodstream from where travels

to the target tissues.

In the present study, a 34 kD protein (previously designated $2B_9$) has been identified as a major product of chorionic and allantoic membranes. Results of NH_2-terminal amino acid sequence analysis revealed this protein was 96.8% homologous to human pro-α-1 type III collagen. The biomechanical properties of collagen and its functions as a stabilizing scaffold of connective tissues for the organism have historically been of research interest. Recent evidence has demonstrated that differentiation and behavior of cells during embryonic development are not only controlled by humoral factors but also by interactions with constituents of the surrounding extracellular matrix (Hay, 1984). Study of the role of extracellular matrix in the process of embryo implantation indicated that reconstituted collagen gels were able to support embryo attachment and outgrowth (Jenkinson and Wilson, 1973). A recent report also provided evidence that types I, II, III, IV, and VI could effectively support embryo attachment and outgrowth *in vitro* (Carson et al., 1988). Investigation of the relationship between the extracellular matrix and hormone production by trophoblast showed that embedding trophoblast explants in three dimensional type I collagen gel was responsible for a much higher and longer lasting production of hormones and trophoblast proteins compared to unembed cultures (Castellucci et al., 1990). Type III collagen was thought to be "fetal" collagen at one time

because it was found to be predominant at early stages of embryonic development. Many investigators presumed it to be the reticulin, a compound of fibrous structure containing collagen type III, fibronectin and additional non-collagenous glycoproteins, described by histochemical methods (Kleinman et al, 1981; Unsworth et al., 1982). These data suggest an important role of type III collagen in embryonic development. In the present study we have isolated a major product of bovine chorioallantois, identified it as the carboxyl-terminal propeptide of bovine α-1 type III collagen, cloned cDNA of the protein and sequenced the clone.

2. Materials and Methods

A. Animals

Beef cows (primarily Angus X Hereford) were observed for estrous behavior, synchronized by a single i.m. injection of prostaglandin F2α (Lutalyse, the Upjohn Co., Kalamazoo, MI) and bred by artificial insemination 8-12 h after initially being observed in estrus (day 0). Animals that did not exhibit estrous behavior received a second injection 11 days after the first. Cattle were slaughtered on day 28, 36 and 45 of pregnancy. Reproductive tracts were collected at slaughter, placed on ice, and returned to the laboratory within 30 min.

B. *In Vitro* Culture of Chorionic and Allantoic Membranes

Under sterile conditions, uterine horns were opened, and the conceptus was carefully dissected out. Chorioallantois was isolated by dissection and placed in a 100 X 20 cm sterile petri dish containing Eagle's minimal essential medium (MEM; GIBCO, Grand Island, NY) and cut into 2-3 mm pieces. Approximately 200 mg of tissue were transferred to 60 X 15 mm tissue culture dishes containing 10 ml MEM and 5 μCi/ml [^{3}H]-Leucine (Dupont-New England Nuclear, Boston, MA). Culture conditions were as described by Godkin et al. (1982a). Tissue explants were cultured at 37 °C on a rocking platform (6 cycles/min) in an atmosphere containing 5% CO_2 (by volume). Medium was removed after 24 h under sterile conditions and clarified by centrifugation at 12,000 X g for 20 min. Tissue explants were replenished with fresh MEM and L-[^{3}H]leucine and incubated for a second 24 h period; the medium was obtained by centrifugation as above. Medium was stored at -20 °C.

C. Two-dimensional SDS Polyacrylamide Gel Electrophoresis (2D-SDS-PAGE)

Medium from Day 28 to 45 chorionic and allantoic membrane cultures were pooled (approximately 500 ml) individually and concentrated to about 25 ml of final volume using a Spectra/Por stirred cell apparatus (Mr cut-off = 10,000). Low molecular weight compounds (salts, phenol red etc.) were removed by dialysis (Mr cut-off = 6,000-8,000) against 10 mM

Tris-HCl (pH 8.0), 0.02% (w/v) NaN_3, 1 mM PMSF (phenylmethylsulfonyl fluoride) for 48 h with four changes. PMSF was not added after the first change, Tris-HCl concentration was lowered to 2 mM at the last change. Two-dimensional PAGE was performed according to the method of Horst and Roberts (1979). Briefly, aliquots of dialyzed medium were lyophilized, dried and dissolved in electrophoresis buffer containing 5 mM K_2CO_3, 9.2 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Radioactivity of dissolved sample was determined by scintillation spectroscopy. Aliquots (about 200 μg) containing 200,000 cpm of the sample were subjected to isoelectric focusing (IEF) gel in the first dimension in 4% (w/v) acrylamide gels containing N,N' diallyltartdiamide, 8.0 M urea, 2% (v/v) Nonidet P-40 and 5.1% (v/v) ampholines (pH 3-10, 5-7, and 9-11; 25:18:8 by volume respectively) (Pharmacia, Uppsala, Sweden). After electrofocusing, tube gels were then equilibrated in 0.07 M Tris-HCl buffer (pH 6.8) containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and subjected to electrophoresis in the second dimension using 12.5% (w/v) polyacrylamide slab gels and the buffer system of Laemmli (1970). Slab gels were fixed and stained with Coomassie blue R-250, impregnated with 1 M sodium salicylate for 30 min, and dried. Fluorograms were prepared by exposing Kodak XAR film to impregnated dried gels for about 3 weeks at -80 °C (Chemical supplies, except where specified, were all from Sigma Chemical Co. St. Louis, MO).

D. N-Terminal Protein Microsequencing

After 2D-SDS-PAGE, slab gels containing proteins to be sequenced were electrotransblotted onto polyvinylidene difluoride membranes (PVDF; Immobilon; Millipore Co., Bedford, MA) for 1 h at room temperature at 200 mA using a modified procedure of Matsudaira (1987). The membranes were rinsed with H_2O briefly and proteins stained with 0.1% Coomassie Blue R250 (Serva, Inc., Westbury, NY) for about 30 second. The protein spot corresponding to $2B_9$ was excised from the membrane. N-terminal amino acid analysis was carried out by gas-phase Edman degradation on an Applied Biosystems 477A sequencer (Applied Biosystems Inc., Foster City, CA). Obtained amino acid sequences were analyzed using the University of Wisconsin, Genetics Computer Group Sequence Analysis Programs (Devereux, 1984) to determine amino acid sequence identity with amino acid sequences of known proteins.

E. Probe for Screening cDNA Library

Polymerase chain reaction (PCR) was performed to construct a probe for cloning $2B_9$ cDNA. The modified pBluescript SK polylinker and sequence primer (TCTAGAACTAGTGGATC, Stratagene, La Jolla, CA) was used as 5' end primer, and a 20 bp oligo nucleotides [sequences designed according to the 3'region of human pro-α-1 type III collagen mRNA (nucleotide bases 4196 - 4215: 5'CCCAGAACATCACATATCAC3'), Ala-Kokko, 1989] was used as 3' end primer. Using the method

described by Sambrook, et al. (1989), bovine conceptus cDNA library (1 μg) was prepared for PCR amplification by adding the above primers, TAQ polymerase (Promega, Madison, WI), and reaction buffer, according to manufacturer's recommendation. Reaction volumes of 50 μl were subjected to thermal cycling amplification in an Precision Scientific thermal cycler (Precision Scientific Inc., Chicago, IL) as following: 1 min at 94 °C, 1 min at 56.5 °C, and 1 min at 74 °C for 35 cycles. Amplified products were then size fractionated on a 1 % agarose gel and visualized by ethidium bromide staining. Two major PCR products were purified on the agrose electrophoresis gel. The higher molecular weight product which was about 600 bp long was released from the gel by β-agarose I (BioLab, New England), the released DNA was subjected to double strand DNA sequencing using +SK primer (Stratagene) as the sequencing primer (United States Biochemical, Cleveland, Ohio). The sequence homology search showed that the 600 bp PCR product had 83% identity with human pro-α-1 type III collagen.

F. cDNA Library Screening And Nucleotide Sequencing

The bovine conceptus cDNA library was established from polyadenylated mRNA isolated from whole conceptuses obtained on days 18 and 21 of pregnancy and chorioallantoic membranes prepared on days 29 and 33 pregnancy (Liu, 1993). The cDNA library was constructed in the λ-ZAP-XR cloning system (Stratagene), and was plated at a density of 50,000 plaques

per 150-mm Petri dish with Escherichia coli XL1-Blue as the host cell. After growth at 37 °C for 6 h, plaques were transferred onto nitrocellulose filters (Gelman & Sciences, Ann Arbor, Michigan), and were lysed in 1.5M NaCl, 0.5M NaOH for 2 minutes, neutralized for 5 minutes by submerging in 1.5M NaCl, 0.5M Tris-HCl, pH 7.4, rinsed in 0.2M Tris-HCl, pH 7.5, 2X SSPE for 30 seconds. Dried filter membranes were prehybridized in 2.5mM EDTA, 50% formamide, 5X Denhardt's, 0.1% SDS, 0.1 mg/ml salmon sperm DNA at 65 °C for 4 h. For hybridization, the 600 bp PCR product was random labeled by ^{32}P-dCTP and added to the hybridization buffer. Filter membranes were hybridized at 42 °C for 24 h. Filters were washed twice for 15 minutes in 5X SSPE with 0.1% SDS at 42 °C. The second wash was in 1X SSPE with 0.1% SDS for 15 minutes at the same temperature as the first wash. The third wash was in 0.1X SSPE with 0.1% SDS for 15 minutes, again for 30 minutes at 65 °C. About 250,000 recombinant phage plaques were screened, positive plaques were selected and rescreened twice. Of 40 resulting positive clones, two clones were isolated and rescued from λ-ZAP-XR into the M13 phagemid (pBluescript SK-) as described by manufacturer's instructions (Stratagene). Single strand DNA was isolated from the M13 phage of SK- phagemid. The nucleotide sequence was determined by the dideoxy chain termination method (Sanger, 1987) using either single-stand or double stand dideoxy chain termination procedures (United States Biochemical, Cleveland, Ohio).

Complete sequencing was achieved by primer walking using oligonucleotide based on already determined bovine sequences. Sequence analysis and homologies were determined by utilizing the University of Wisconsin, Genetics Computer Group Sequence Analysis Programs (Devereux, 1984).

3. Results

A. Identification of $2B_9$ protein

As demonstrated in the fluorogram depicted in figure 1, protein $2B_9$ is one of the major protein synthesized and secreted by chorioallantois in culture. The protein had an estimated mass of 34,000 and a pI of 5.5. It tended to be produced more in allantois than in chorion culture medium (fig. 2).

The protein was isolated by 2D-SDS-PAGE, electrotransblotted onto a PVDF membrane and subjected to NH_2-terminal amino degradation microsequencing. After 35 cycles of Edman analysis, the first 31 NH_2-terminal amino acids of $2B_9$ were identified. Compared to the known protein sequences, the first 31 amino acids of $2B_9$ shared 96.8% identity with human pro-α-1 type III collagen, 87.1% identity with rat α-1 type III collagen, 56.1% identity with chicken α-1 type III collagen (fig. 3).

B. Isolation and Characterization of the $2B_9$ cDNA clone

The bovine conceptus cDNA library was screened using a 600 bp PCR product with 83% identity to human pro-α-1 type III collagen. Two positive clone were finally isolated from 40 original positive clones and selected for DNA sequencing. The clones were sequenced twice from both ends, and a consensus sequence was obtained. Figure 4 shows that the nucleotide sequence of the longer clone, designated 9.22, is 1829 bp in length. The largest open reading frame of the cDNA starts at nucleotide base 1 and ends at base 885, and the 3'-untranslated region (3'-UTR) contains 944 nucleotide bases. A computer database search revealed clone 9.22 shared 86.9 % nucleotide sequence homology and 93.5 % of deduced amino acid sequence homology with the carboxyl-terminal of human pro-α-1 type III collagen (fig. 5). Further proof that the cDNA clone was indeed coding for the α1 chain of type III procollagen resulted from the identification of two successive cysteine residues and the presence of only two G-P-P tripeptide units at the carboxyl-terminal end of the triple helical domain which were determined as two unmistakable features of type III collagen chains by Seyer (1981). The deduced amino acid sequence contained one potential carboxyl-terminal peptidase cleavage site (YYG/DEP) between residues 50 and 51. The first 31 residues downstream from the cleavage site were in agreement with the NH_2-terminal amino acid sequence obtained from Edman degradation analysis. A single tripeptide sequence

(N-I-T), a potential carbohydrate attachment site, was found at residues 195-197. The difference between the calculated MW of $2B_9$, 27.3 kD, with the actual MW showed on the 2D-SDS-PAGE, 34 kD, could be caused by additional attachment of a carbohydrate chain. There were 5 AATAAA polyadenylation sites (two which overlapped) in the 3'-untranslated region. Assuming that D (Aspartic acid) is the first amino acid of the $2B_9$ protein, the entire coding region of the protein contained 245 amino acid residues, of which eight cysteine residues were found in the region. Nucleotide and deduced amino acid homologies between cow, human, rat and chicken procollagen III are presented in fig. 5.

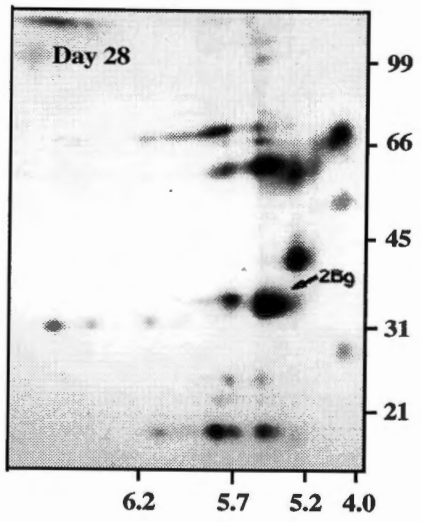

Fig 1. Fluorogram of 2D-SDS-PAGE of dialyzed medium protein from day 28 bovine chorioallantois cultured in MEM with the presence of [^{3}H]-leucine for 48 hr. An aliquot of medium protein (200,000 cpm) was separated in the first dimension by isoelectric focusing, then separated in the second dimension on a 12.5% (w/v) polyacrylamide gel in the presence of a reducing agent. The vertical axis on the side of the gel indicateds molecular weight (X 10^{-3}), the horizontal axis at the bottom side of the gel indicates isoeletric point (pI). Note that $2B_9$ was a major radiolabeled protein with an estimated mass of 34 kD and pI of 5.5.

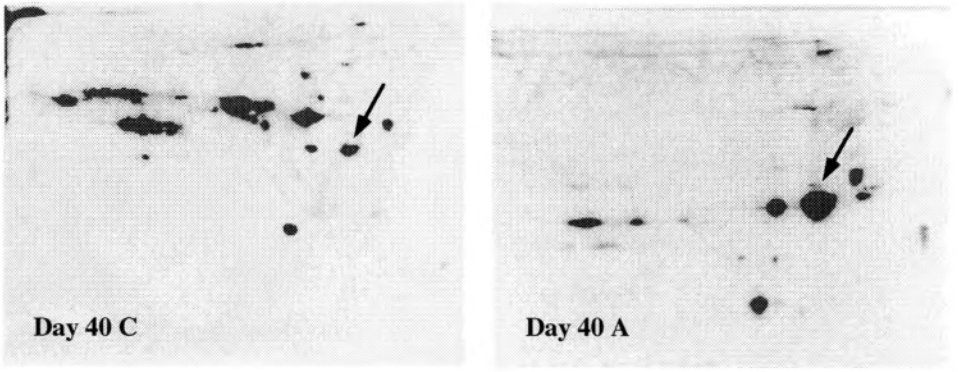

Fig 2. Two-dimensional SDS-PAGE analysis of synthesized proteins from day 40 bovine chorion (left) and allantois (right) cultures. Culture condition and 2D-SDS-PAGE running prodedure were same as described in fig 1. Equal amounts (200,000 cpm) of solubilized proteins were loaded in each gel. Note that $2B_9$ production was predominantly from allantois.

```
Bovine α1(III)    DEPIDFKINTDEIMTSLKSVNGQIESLISPD
Human α1(III)        M
Rat α1(III)        D M      E   S
Chicken α1(III)      KENE  LG   S M  I N   NIL
```

Fig. 3. Comparison of NH_2-terminal amino acid sequences (single letter abbreviation) of protein $2B_9$ to human, rat, and chicken sequences. The relative sequence homologies between bovine $2B_9$ to human, rat, and chicken procollagens are 96.8%, 83.8%, and 54.8% respectively. Only the amino acid residues different from the bovien sequence are shown.

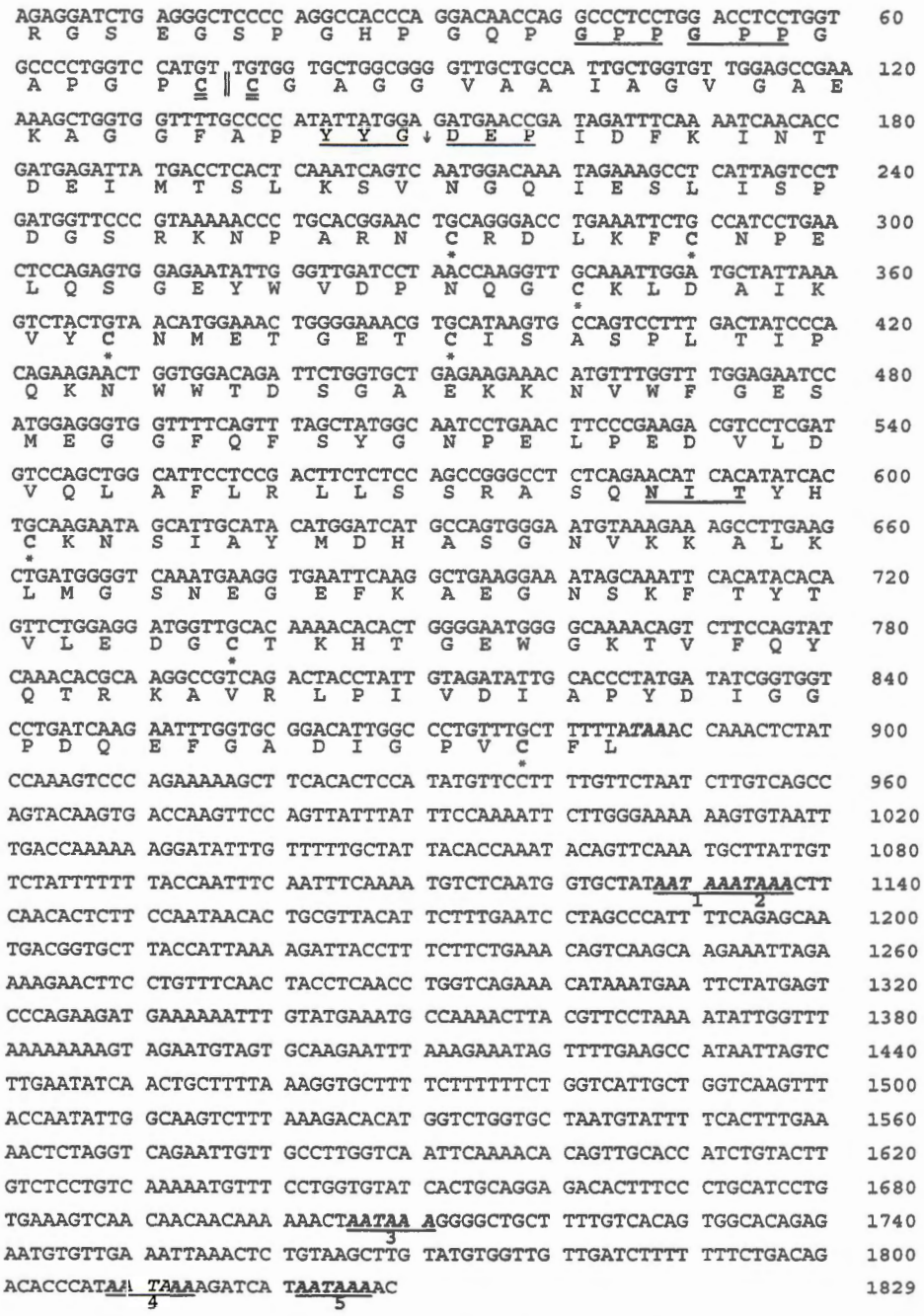

Fig 4. Bovine C-propeptide of collagen III cDNA sequences and deduced amino acid sequences. Important amino acid and nucleotide sequences are underlined. Two (G-P-P) units and (C‖C) indicate the end of the triple helical domain and the beginning of C-propeptide domain. The remaining cysteinyl residues are signified by (*) underneath. Tripeptide (N-I-T) shows the potential carbohydrate attachment site. The symbol (↓) identifies the potential C-propeptide cleavage site. The first in-frame translation codon is indicated by (***). Five polyadenylation sites are numbered underneath. Sequences from symbol (‖) to (↓) indicate the location of C-telopeptide.

species	collagen	nucleic acid	amino acid
Human	*procollagen III*	*86.9 %*	*93.5 %*
*Rat**	*collagen III*	*83.5 %*	*87.9%*
Chicken	*3'collagen III*	*53.3 %*	*63.7 %*

Fig. 5. Bovine C-propeptide of collagen III sequence compares to collagens of other species (percent of homology for coding sequences). * Note that reported rat sequence is not completed.

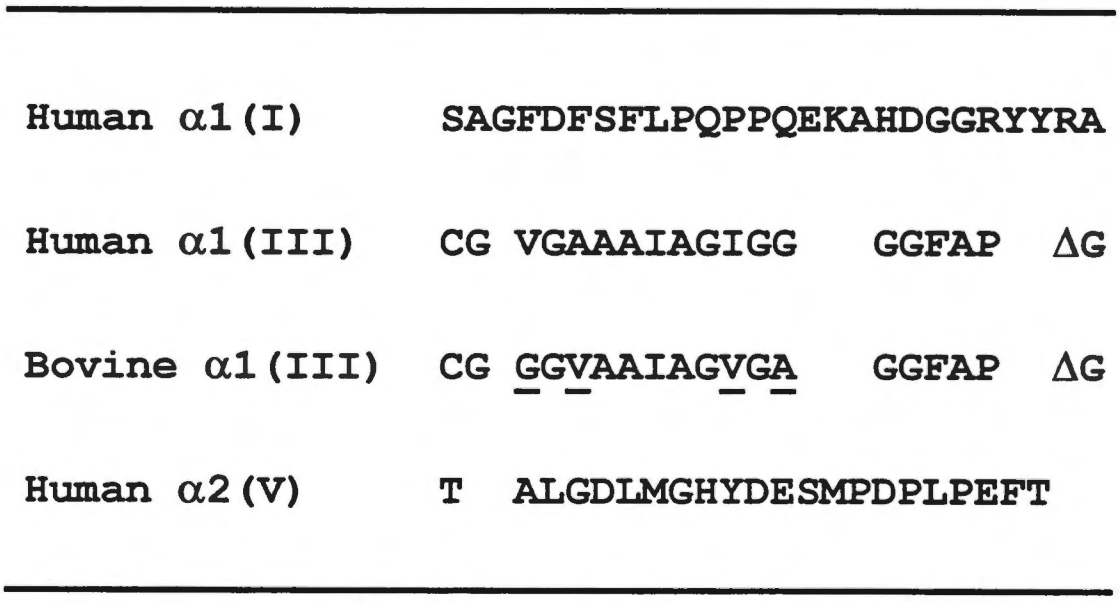

Fig 6. Amino acid sequences of C-telopeptide of bovine α1(III) procollagen and human α1(I) (Bernard et al., 1983b), α1(III) (Loidl et al., 1984; Chu et al., 1985b), and α2(V) (Myers et al., 1985; Weil et al., 1987) procollagen. Only the residues different from human α1(I) sequence are shown. Residues in bovine α1(III) chain that are different from human α1(III) chain are underlined. "Δ" represents lack of corresponding amino acid residue.

4. Discussion

Collagen represents a major group of proteins in mammals and plays an essential role in the structure and function of most connective tissues. At least 18 genetically and biochemically distinct types of collagen have been identified in different tissues (Rehn and Pihlajaniemi, 1994). Type III collagen is a member of fibrillar collagen family (Miller, 1988). The polypeptide chains of the fibrillar collagens are synthesized from procollagens, precursor molecules which are each composed of three pro-α chains. Beginning at the amino terminal end, they contain a signal peptide, an amino-terminal propeptide (N-propeptide) domain, the triple helical domain of the protein with the repeating Gly-X-Y motif and a carboxyl-terminal propeptide domain (C-propeptide). The signal peptide is removed within the cell, whereas the amino and carboxyl propeptides are cleaved extracellularly by specific amino- and carboxyendopeptideases before the mature proteins undergo the process of fiber formation (Bornstein and Sage, 1980). Alterations in the structure, synthesis, or processing of these proteins in human may result in a variety of inherited disorders, such as osteogenesis imperfecta, chondrodystrophy, Marfan syndrome, and Ehlers-Danlos syndrome (Prockop, et al., 1979). Some indirect evidence showed that the C-terminal propeptide is involved in the alignment of the triple helix. Thus, the interchain disulfide bonds located in C-propeptide of type I collagen is formed before the triple

helix formation occurs (Uitto and Prockop, 1973). Administration of low concentration of puromycin to chicken tendon fibroblast cultures, resulted in shortening of the synthesized pro-α chains largely due to inhibition of production of the C-terminal extension. These pro-α chains were not triple-helical and were not secreted (Rosenbloom et al., 1976). Capaldi and Chapman (1982) suggested a model which both NH_2- and C-telopeptides participate in linear assembly of fibrils and the C-propeptide participates in lateral assembly resulting in the periodic arrangement of the collagen fibril. To support this model, they combined specific enzyme digestion and electron microscopy experiments which demonstrated that a specific folding in C-telopeptide of type I collagen played a functional role in fibril assembly.

In the present study we report that a major secreted protein, designated $2B_9$, produced by bovine chorioallantois, was identified as the C-propeptide of α-1 type III collagen. Comparison of the cDNA sequence of this C-propeptide with other C-terminal propeptides of fibrillar collagen genes revealed that strong similarities have been maintained between species and different procollagen chains. First, the carboxyl propeptides of fiber forming collagens are homologous in length. Bovine C-propeptide of α1(III) collagen ($2B_9$) contains 245 amino acid residues, identical to that of human C-propeptide of procollagen III (Loidl et al., 1984; Chu et al., 1985b), while avian consist of 246 amino acid residues

(Yamada et al., 1983). All the four α chains of human α1(I), α2(I), α1(II) and α1(III) studied are 245 to 247 residues in length (Dion, 1987). The C-propeptide of collagen III from bovine, human and avian species all contain eight cysteine residues (Kühn 1987). Data from Dayhoff (1978) showed that cysteine represents one of the most highly conserved amino acid residues among evolutionarilly related proteins. Therefore, it is not surprising that cysteine is maintained as a conserved component of the C-propeptide of bovine procollagen III. According to Vuorio and de Crombrugghe (1990), four cysteines in the C-propeptide are thought to be involved in intrachain disulfide bridges, the other four cysteines residues form interchain bridges between the three α chains. The interchain bridges are especially important for the formation and stabilization the globular structure of C-propeptide. The bulky globular structure of the propeptides of the intact collagen molecule may serve as a recognition site for the triple-helical trimmer assembly, and may also function as a transport form and prevent pre-mature fibril secretion or assembly inside the cell.

Secondly, the locations of the cysteine residues and their neighboring sequences in the C-propeptides are strictly conserved as well as the sequence around the carbohydrate attachment site (Myers and Dion, 1990). Sequence N-X-T/S, in which X maybe any amino acid except proline or aspartic acid, is required for N-asparaginyl-linked glycosylation and is

commonly referred to as a potential carbohydrate attachment site. This particular interchain and inter-species sequence conservation has been identified in human α1(I), α2(I), α1(III), α2(V) (Bernard, 1983b, 1983a; Chu, 1985b; Myers, 1985) and avian α1(I), α2(I), α1 (II), α1(III)(Fuller, 1981; Sandell, 1984; Ninomiya, 1984; Yamada, 1983), suggesting conservation of important functional sites in these regions. The importance of adding carbohydrate chains to the carboxyl end of procollagen is still unclear, perhaps the carbohydrate chains contribute to a recognition mechanism for procollagen alignment, secretion, or assembly into microfibril.

A third homologous feature found in fibrillar collagens is the presence of multiple polyadenylation sites (AATAAA hexanucleotides; Proudfoot and Brownlee, 1976) in the 3'-UTRs of their genes. In clone 9.22, it was found that the bovine procollagen III gene contained five polyadenylation sites (two were overlapped) in the 944-nucleotide sequence of 3'-untranslated region. The two overlapped polyadenylation sites [poly (A) sites] were located at +243 and +247 bp downstream of the translation termination codon, TAA (+1 designates the first nucleotide of the termination codon). The other three poly (A) sites were closely positioned at the very 3' end of the gene, +821, +924, and +937 bp respectively. 3'-UTR of human procollagen III gene contains a total of 960 nucleotide bases (Ala-Kokko et al., 1989). Four poly (A) sites were found at similar position as above, +254, +258 (overlapped), +835,

and +952 bp. The poly (A) site at position +924 bp in bovine seems missing in human. Other fibrillar collagen genes also contain multiple poly (A) sites such as human α1(I) collagen contains three poly (A) sites (two are overlapped) (Chu et al., 1985a), human and avian α2(I) collagens contains four poly (A) sites (Aho et al., 1983; Myers et al., 1983; and Fuller et al., 1981), and human α1(II) collagen which contains two poly (A) sites (one is variation of the AATAAA hexanucleotides) (Stoker et al., 1985). It seems the presence of multiple AATAAA hexanucleotides is a characteristic of this gene family.

Although the C-propeptide regions of the fibrillar collagen α chains are strongly homologous to one another, there must be characteristic sequence regions in the propeptides which determine the type-specific interactions of the C-propeptide. Interestingly, the 3'-UTRs also represent diverging regions among different types of collagens. The length of the 3'-UTRs is different in different collagens (Chu, 1985b; Myers, 1983; Ala-Kokko, 1989), the number and position of poly (A) sites are different also (Myers, 1986). Calculation of sequence difference K (the number of mismatches per nucleotide site in a pair of aligned sequences) indicated that a strong evolutionary selective pressure is exerted upon the untranslated regions (Miyata, 1980). In collagen, the above divergent evidences in 3'-UTRs may be the characters in this case.

Another variable region is located at the C-telopeptide (residues 26-50, fig. 4, from marks "‖" to "↓"). Comparison of this region of bovine C-propeptide III to the region in human α1(I), α1(III), and α2(V) C-propeptides (Weil, et al., 1987) (fig. 6), demonstrates that the bovine sequence has very low sequence homology with human α1(I), and α2(V) sequences, while only four amino acid residues are different between human α1(III) and bovine α1(III) C-telopeptides. Similar comparative studies of the avian α1(I), α1(II) and α1(III) C-telopeptide performed by Sandell et al. and Yamada et al.(1984; 1983) revealed a high degree divergence between the procollagen genes examined. The C-telopeptide may play a role specific to the individual collagen chains and contribute to produce characteristic types of collagen fibers.

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CHAPTER VI

Developmental Gene Expression of Procollagen III in Bovine and Ovine Extraembryonic Membranes during Early Pregnancy

1. Introduction

Collagen is the major component of matrix proteins and comprises a large heterogeneous class of protein molecules, of which at least 18 different types have been identified (Rehn & Pihlajaniemi, 1994). It has been determined that cell differentiation, proliferation, migration, organization, and attachment during embryonic development are controlled by cellular interactions with constituents of the surrounding extracellular matrix (Hay, 1991). Various cells synthesize different collagens and modify them to produce structural matrices specific to the cell type. Consequently, collagen expression is carefully regulated during differentiation and embryogenesis (von der Mark, 1976; Linsenmayer & Toole, 1977; Bornstein & Sage, 1980). Types I, III, IV, V, and VI collagens have been found in the utero-placental unit of humans, mice and cattle (reviewed in Sharpe et al., 1990). Failure to correctly express or modify specific collagens have been implicated as the causative factor in several diseases of man and animals (Prockop, 1979; Bornstein and Byers, 1980).

In cattle and sheep, after onset of estrus (day 0), fertilization occurs on day 1 (Hunter, 1985). Around day 6, the embryo develops into a blastocyst which is composed of

inner cell mass, the surrounding trophectoderm and the blastocoele. The blastocyst hatches from the zona pellucida around day 10-12 (Renard, 1978). Over the next 10 days the blastocyst undergoes tremendous growth. By day 17 in sheep and day 24 in cow, the chorionic and allantoic membranes are visible, the conceptus fills the gravid uterine horn and extends into the contralateral horn (Bazer and First, 1983). Between days 20 and 28, the allantois undergoes dramatic expansion or elongation, and then fuses with chorion, forming the chorioallantois (Greenstein, 1958). During the next 30-40 days the chorioallantois and endometrium form an interdigitating complex, placenta.

Clearly, the remarkable growth and development of the ruminant conceptus requires involvement of different functional molecules produced by both the embryo and endometrium. Many of the functions necessary for embryonic development have been attributed to the extracellular matrix proteins. 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 (Green, 1968). A *Drosophila* collagen gene has been shown to be expressed in a developmentally regulated manner (Monson, 1982). In the chicken embryo, the RNAs coding for types I, II, and III collagen accumulated dramatically between day 5 and day 15 of development (Merlino, 1983).

In our previous study, we described a major secreted

protein (previously designated $2B_9$) isolated from day 28-45 bovine extraembryonic membrane (chorioallantois) culture medium. This was identified as the carboxyl-terminal of procollagen III. DNA sequencing of the $2B_9$ clone showed there were multiple polyadenylation sites present at the 3'-untranslated region (3'-UTR). Here we report our further study of developmental gene expression of bovine and ovine procollagen III during early embryonic development and the differential usage of at least two polyadenylation sites.

2. Materials and Methods

A. Materials

Guanidine isothiocyanate (GTC) was prepared by the method described by Sambrook et al. (1989). Cesium trifluoroacetate (CsTFA) was purchased from Pharmacia (Piscataway, NJ) and prepared for RNA isolation by isopycnic gradient ultracentrifugation according to manufacturer's instructions. Agarose was obtained from Baxter Scientific Products (McGraw Park, IL). Electrophoretic buffer reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Formamide, formaldehyde, Random Prime labeling and Sequenase DNA sequencing kits were from United States Biochemicals (Cleveland, OH). α^{32}P-dCTP (3,000 Ci/mmole) was obtained from New England Nuclear (Du Pont, Boston, MA). RNA molecular weight standards were purchased from IBI (International Biotechnologies, Inc., New Haven, CT). Nytran, positively charged nylon membranes were

purchased from Schleicher and Schull (Keene, NH). Low-melting point (SeaPlaque) agarose was from FMC Bioproducts (Rockland, MA) and gelase was from EpiCenter Technologies (Madison, WI). Chemical supplies for tissue culture were purchased from Sigma Chemical Co. St. Louis, MO. Other buffer reagents and diethylpyrocarbonate (DEPC) were from Baxter Scientific Products.

B. Tissue Collection

The estrous cycles of crossbred beef cows (primarily Angus X Hereford) were synchronized by a single i.m. injection of prostaglandin F2α (Lutalyse, The Upjohn Co., Kalamazoo, MI) and bred by artificial insemination 8-12 h after initially being observed in estrus (day 0). Animals that did not exhibit estrous behavior received a second injection 11 days after the first. Cattle were slaughtered on days 17, 20, 24, 26, 29, 36 and 45 of pregnancy. Reproductive tracts were removed rapidly (within 30 min after death) and flushed with 40 ml MEM into a petri dish to collect bovine conceptus tissues.

Adult crossbred ewes were checked daily in estrus (designated day 0) with vasectomized rams. Ewes demonstrating estrus behavior were bred with an intact ram or allotted to the cyclic group. Pregnant ewes were anaesthetized (methoxyflurane) and the reproductive tract was exposed surgically via medventral laparotomy. Ovine conceptuses were flushed from uteri on day 13, 16, 20 of pregnancy with sterile

MEM at 37 °C and collected in sterile serum bottles. Alternatively, day 23, 27, and 30 pregnant ewes were hysterectomized and the chorionic and allantoic membranes ewes were dissected out of the uterus under sterile condition.

C. RNA Isolation

Collected individual conceptuses or chorioallantois was rinsed with ice cold 0.9% (w/v) NaCl and homogenized in ice cold guanidine isothiocyanate (GTC). Total cellular RNA was recovered by subjecting the homogenate to isopycnic gradient ultracentrifugation on a cushion of CsTFA. The supernant was carefully removed after centrifugation, the RNA pellet was resuspended in 30 mM sodium citrate (pH 7.4), 0.1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol, and washed with ethanol to remove residual cesium. Precipitated RNA was dried at room temperature and dissolved in a buffer containing 1X MOPS (40 mM morpholinopropane sulfate, 10 mM sodium acetate, 1 mM EDTA, pH 8.0) with 0.1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. Concentration of the RNA preparation was determined by measuring the OD_{260}, and the preparation was stored at -80 °C.

D. Probes for Northern blots

In a previous study, the cDNA of bovine C-propeptide of collagen III (previously designated $2B_9$) was cloned and designated clone 9.22 (Chapter 3), which was 1829 bp in length and contained 5 polyadenylation sites [poly (A) sites]. In

order to detect the possible usage of multiple transcription termination signals, two probes were designed. Probe A was made to sequences (587 bp - 937 bp) upstream from all the poly (A) sites of template clone 9.22, which was expected to identify all the possible transcripts. Probe B was made to sequences (1233 bp - 1671 bp) between poly (A) sites 2 and 3 (figure 1), which was designed to identify the possible differential usage of poly (A) sites. These two DNA probes were prepared by PCR and random labeled with α^{32}P-dCTP.

E. Northern Blots

Isolated total cellular RNA was electrophoresed in a 1.2% MOPS / formaldehyde agarose gel (10 μg of RNA/lane), capillary transferred onto a Nytran nylon membrane, and immobilized by UV irradiation. Hybridization was carried out in 2.5 mM EDTA, 50% formamide, 5X Denhardt's, 0.1% (w/v) SDS with 100 μg/ml of heat-denatured salmon sperm DNA for 4 h at 65 °C (prehybridization) and 24 h at 42 °C (hybridization) in a hybridization oven (Vangard International, Inc., Neptune, NJ). Membranes were first washed for 15 minutes in 5X SSPE with 0.1 % SDS twice and then in 1X SSPE with 0.1 % SDS once at 42 °C. High stringency washes were in 0.1 X SSPE with 0.1 % SDS for 15 minutes, then again for 30 minutes at 72 °C. Blots were exposed to Kodak Xomat AR film at -80 °C for 16 h. The membrane was boiled in a water bath to remove the probe and rehybridized with a rat β-actin cDNA probe under the same

conditions. With the assistance of an LKB Ultroscan Laser Scanning Densitometer and GSXL version 2.0 software, intensity of signal from each sample band was analyzed by using β-actin autoradiography as a reference of the total quantity of RNA present on the blot.

F. Cloning and Sequencing

To verify the existence of multiple bovine procollagen III transcripts and multiple usage of poly (A) sites, bovine conceptus cDNA was rescreened with the probe previously used for cloning bovine C-propeptide of collagen III under the same conditions (chapter 3). Positive clones obtained from this third round of screening were purified according to the procedure described by Sambrook (1989), and clone sizes were determined by standard Southern hybridization (Southern, 1975) using previous obtained clone 9.22 (chapter 3) as an alternate size reference. Two clones which were smaller than clone 9.22 were selected after Southern blot analysis and rescued from λ-ZAP-XR into the M13 phagemid (pBluescript SK-) as described by manufacturer's instructions (Stratagene, La Jolla, CA). DNA sequencing was performed by either single-stand or double-strand dideoxy chain termination method (Sanger et al., 1977) using the Standard sequenase (R) Version 2.0 Sequencing System kit and protocol.

G. *In vitro* Culture of Bovine and Ovine Chorioallantoic Membranes

In order to determine when bovine and ovine procollagen III were first expressed during early embryonic development, whole conceptuses or chorioallantoic membranes from day 17 through day 45 in cattle and day 13 through day 30 in sheep were cultured and their secreted proteins were analyzed.

Under sterile conditions, collected bovine and ovine conceptus and chorioallantois tissues were placed in 100 X 20 cm petri dishes containing MEM and cut into 2-3 mm pieces. Approximately 200 mg of tissues were transferred to 60 X 15 mm tissue culture dishes containing 15 ml MEM and 5 μCi/ml [^{3}H]-leucine. Culture conditions were as described by Godkin et al (1982). Tissue explants were cultured at 37 $^{\circ}$C on a rocking platform (6 cycles/min) in an atmosphere containing 5 % CO_2 (by volume). Medium was removed after 24 h under sterile conditions and clarified by centrifugation at 12,000 X g for 20 min. Tissue explants were replenished with fresh MEM and [^{3}H]-leucine and incubated for a second 24 h period. Medium was harvested, centrifuged and stored at -20 $^{\circ}$C.

H. Two Dimensional SDS Polyacrylamide Gel Electrophoresis (2D-SDS-PAGE)

Medium from individual tissue cultures were concentrated, dialyzed (Mr cut-off = 6,000 - 8,000) and lyophilized. Dried samples were dissolved in a volume of sample buffer containing

5 mM K_2CO_3, 9.2 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol. Radioactivity of disolved samples were determined by scintillation spectrophotometry. Aliquots 200,000 cpm (about 200 μg protein) of sample were subjected to isoelectric focusing (IEF) in the first dimension in 4% (w/v) acrylamide gels containing N,N' diallyltartdiamide, 8.0 M urea, 2% (v/v) Nonidet P-40 and 5.1% (v/v) ampholines (pH 3-10, 5-7, 9-11; 25:18:8 by volume respectively). After electrofocusing, tube gels were then equilibrated in 0.07 M Tris-Hcl buffer (Ph 6.8) containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and subjected to electrophoresis in the second dimension using 12.5% (w/v) polyacrylamide slab gels and the buffer system of Laemmli (1970). Slab gels were fixed and stained with Coomassie blue R-250, impregnated with 1 M sodium salicylate for 30 min and dried. Fluorograms were prepared by exposing Kodak XAR film to impregnated dried gels for about 3 weeks at -80 °C.

3. RESULTS

A. Developmental gene expression of bovine and ovine procollagen III

Expression of bovine procollagen III mRNA in day 17 and day 20 whole conceptuses and days 24, 26, 29, 36, and 45 chorioallantoic membranes was determined by Northern blot analysis. Hybridization of probe A to day 17 bovine conceptus RNA demonstrated that there was no signal produced more

intense than background. Expression was low at day 20 and increased through day 36 (fig. 2).

Similar gene expression patterns of procollagen III were observed in ovine tissues. Using the same probe (probe A), total cellular mRNA isolated from day 13 and day 16 conceptuses and days 20, 23, 27, and 30 chorioallantois was analyzed by Northern blot. No detectable level of procollagen mRNA was observed at day 13 and 16, expression then increased through day 20 - 27, and declined after the peak at day 27 (fig.3).

Two major transcripts of 5.9 kb and 4.9 kb were identified in both bovine and ovine tissues (fig. 2 and 3). Two dimensional SDS-PAGE of secreted proteins from the same development stages of both bovine and ovine extraembryonic membranes revealed that procollagen III mRNA expression was paralleled by the appearance of the secreted protein after day 20 of pregnancy in cattle and sheep. Fig. 4 and 5 show that the C-propeptide of collagen III was not detectable in protein samples from incubation media conditioned by bovine or ovine conceptuses collected before day 21 of pregnancy.

B. Utilization of multiple polyadenylation sites

Two transcripts were identified by using probe A, of which the 4.9 kb transcript was expressed more prominently than the 5.9 kb transcript. Using probe B, which was made to a sequence between poly (A) sites 2 and 3 of template clone

9.22 (see method), only the 5.9 kb mRNA species was detected on Northern blots of both bovine and ovine tissues (figure 2 and 3).

To further prove the utilization of the multiple poly (A) sites, the bovine cDNA library was rescreened with the probe previously used for isolating clone 9.22. All of the isolated 59 positive clones obtained were smaller than the previous obtained clone 9.22 (chapter 3) as determined by Southern blot analysis. Two clones designated 9.29 and 11.7 were partially sequenced from both ends to a point where there was a 200-300 bp sequence overlapped with the previously sequenced clone 9.22 at each end. DNA sequence analysis revealed that clone 9.29 and clone 9.22 ended at the same place, but clone 9.29 began at 251 bp downstream from 5'end of clone 9.22 (figure 6). Clone 11.7 began at 127 bp downstream from 5'end of clone 9.22 and ended at 672 bp upstream from 3'end of clone 9.22. Clone 11.7 was 1031 bp in length and did not contain poly (A) sites 3, 4, and 5 which were present in clone 9.22 and clone 9.29 (figure 6).

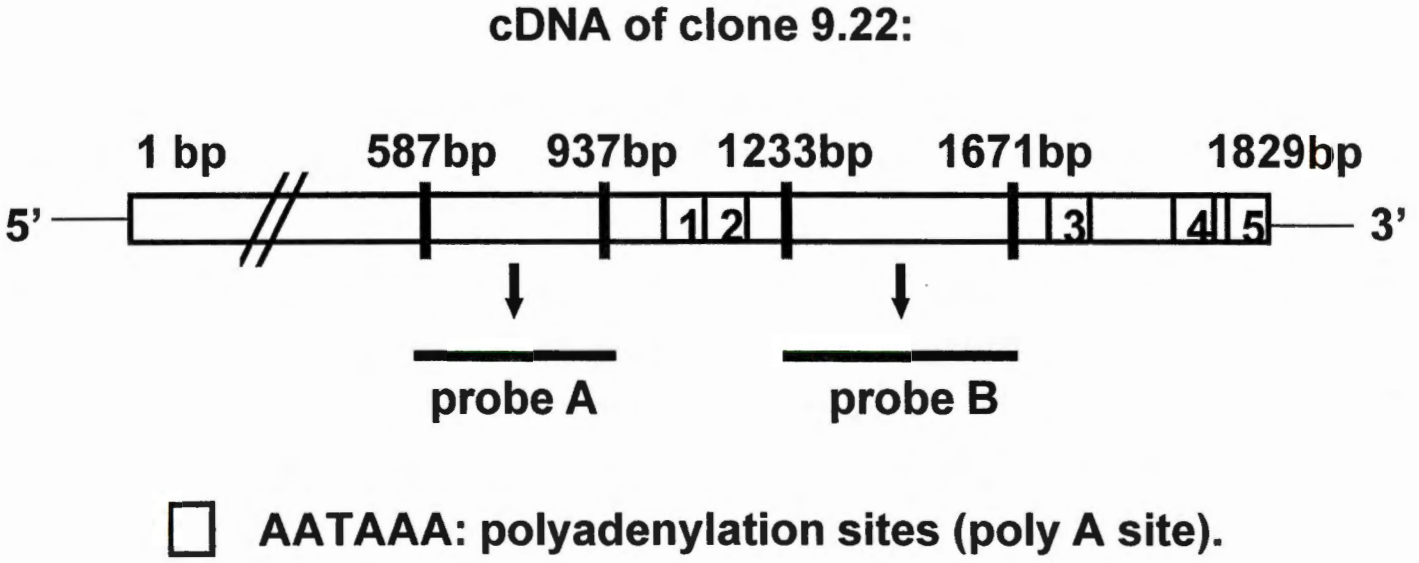

Fig 1. Construction of probes used for Northern blots: probe A was made to a sequence 587 bp - 937 bp upstream from all the poly (A) sites of clone 9.22, probe B was made to a sequence 1233 bp - 1671 bp between poly (A) sites 2 and 3.

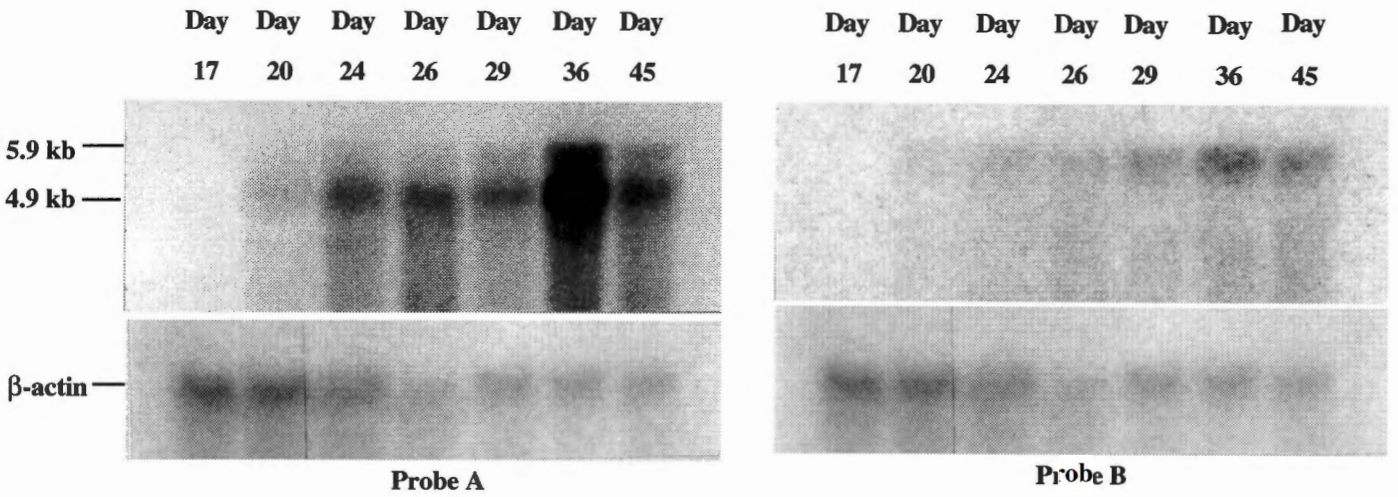

Fig 2. Northern blot analysis of procollagen III mRNA expression in bovine conceptuses and chorioallantois. Total RNA (10 μg/lane) isolated from each day of pregnant cow was fractionated on a MOPS/formaldehyde-1.2% agarose gel, transferred to nylon membranes, and probed with ^{32}P-labeled probe A (right) and B (left). Developmental progress is expressed in days at the top of the autoradiograms. The size of the RNA species was determined by runing standard RNA markers in a paralled lane and visualized with ethidium bromide staining. β-actin mRNA expression was performed to indicated loading and transfer equity between samples.

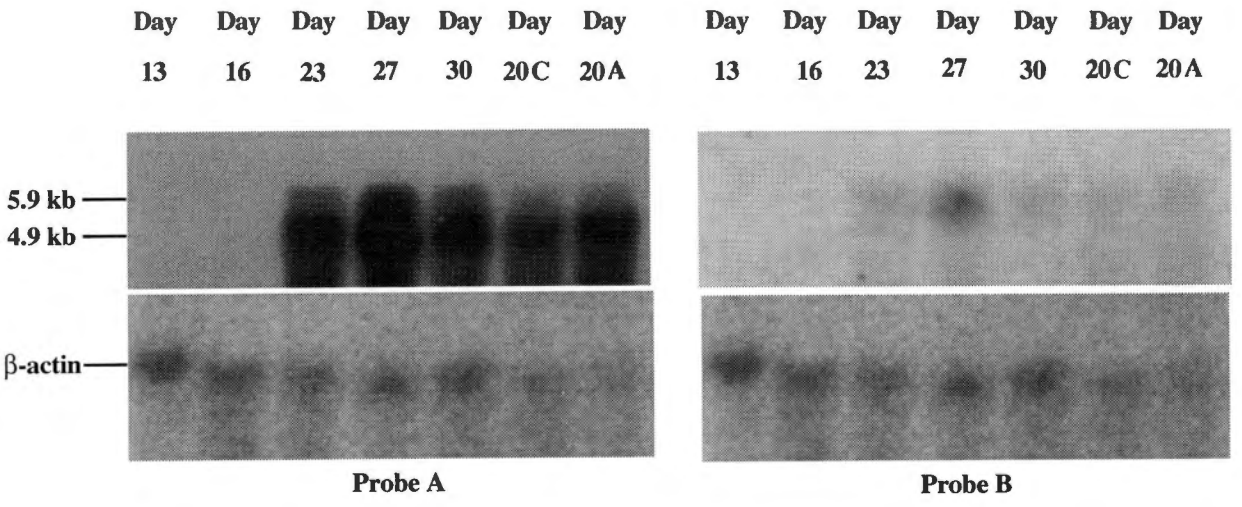

Fig 3. Northern blot analysis of procollangen III mRNA expression in ovine conceptuses, chorion and allantois. Development expression in days is indicated on the top of the autoradiograms. "C" represents chorion; "A" represents allantois.

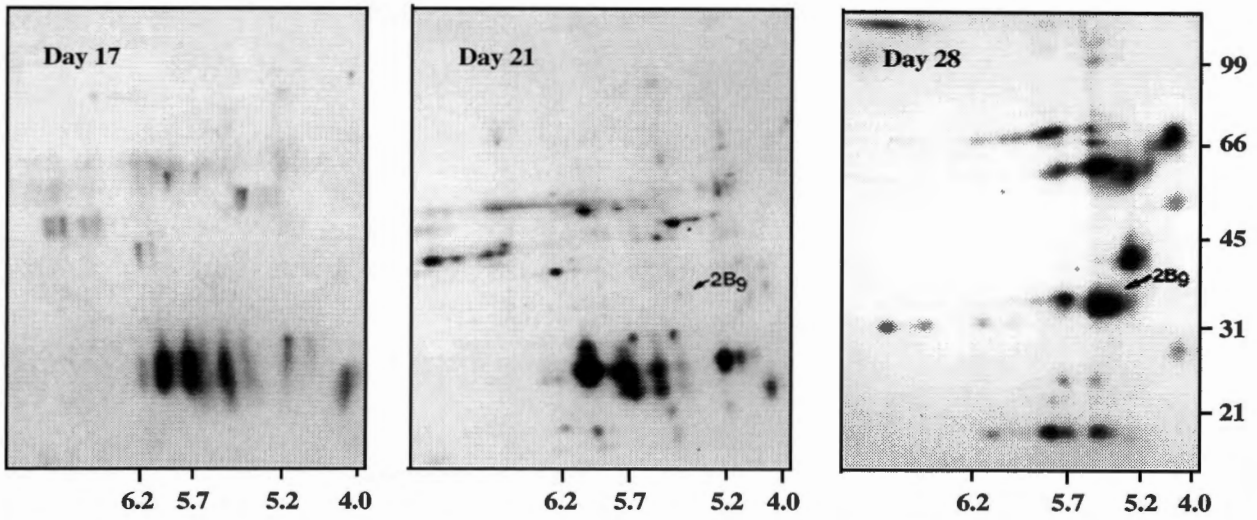

Fig 4. Fluorographs of two-dimensional SDS-PAGE for analysis of radiolabeled bovine proteins released into the medium following culture with the presence of [^{3}H]-leucine for 48 hr. Aliquots of media (200,000 cpm/gel) from day 17, 21 conceptuses, and day 28 chorioallantois were subjected to each gel. Arrow indicates the secretion of bovine C-propeptide of collagen III ($2B_9$).

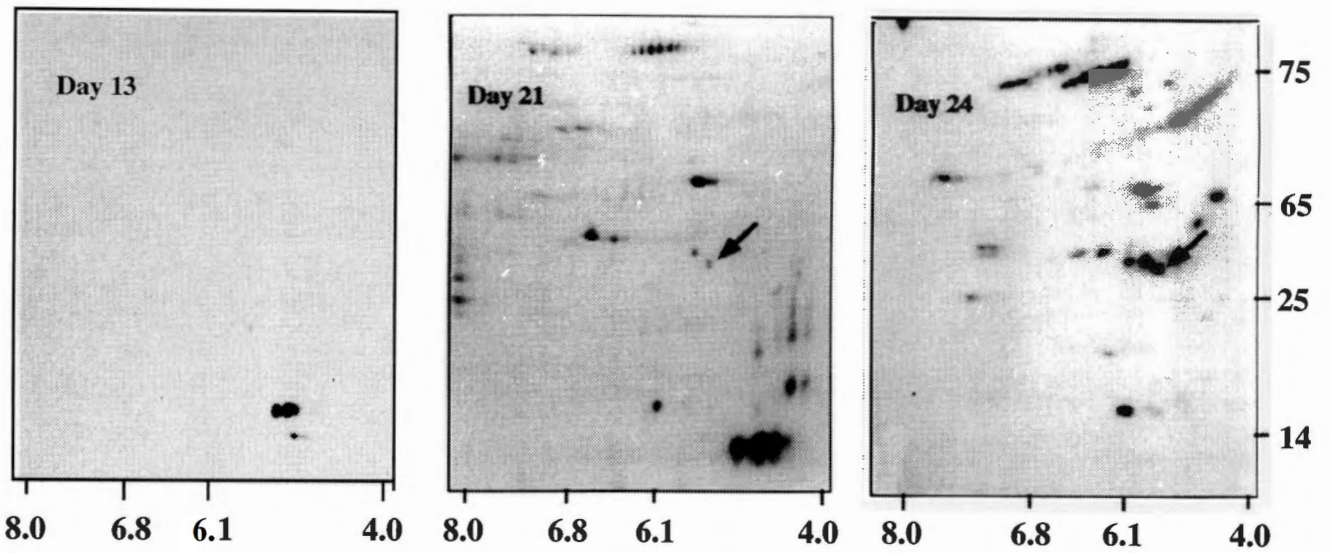

Fig 5. Fluoragraphs of 2D-SDS-PAGE for analysis of ovine proteins released into the medium following culture with the presence of [3H]-leucine for 48 hr. Sample media (200,000 cpm/gel) from day 13, 21 conceptuses, and day 24 chorioallantois were loaded to each gel. Arrow indicates the presence of ovine C-propeptide of collagen III.

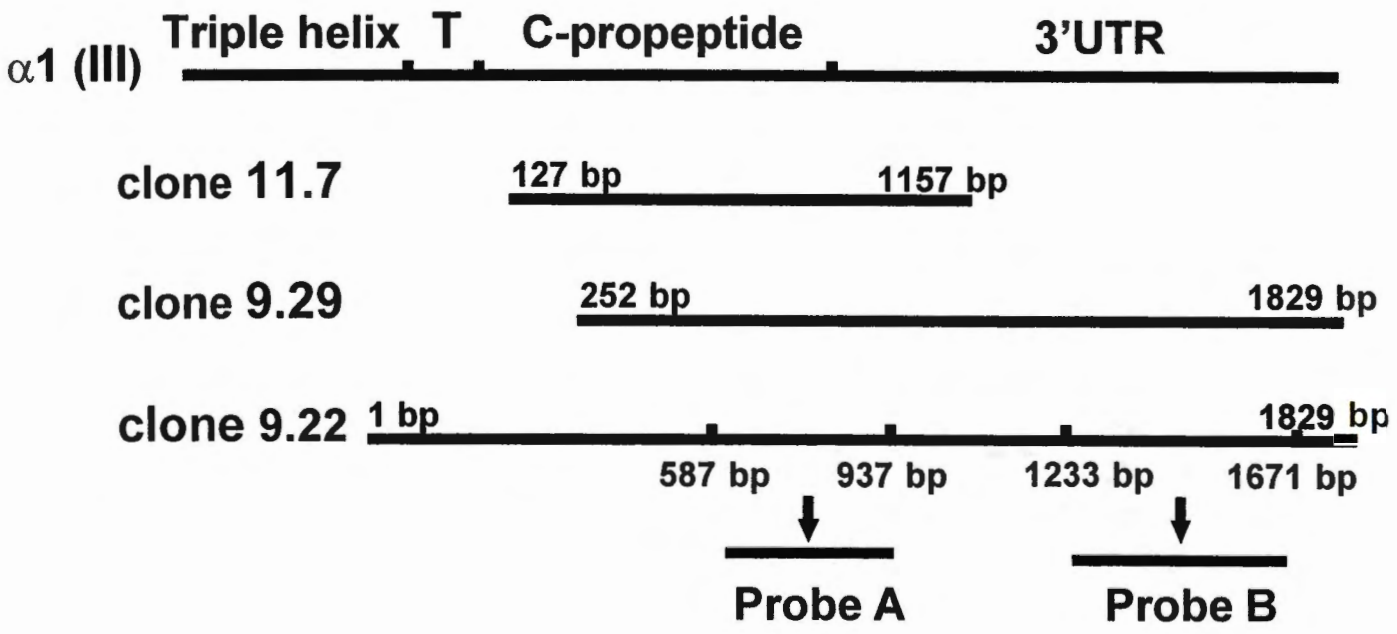

Fig 6. Diagram of comparason of three isolated cDNA clones.

4. Discussion

A. Gene expression of procollagen III

Previously, we identified a protein secreted by bovine chorioallantois which has now been identified as the carboxyl-terminal of procollagen III. The present study was designed to investigate the temporal expression of procollagen III and polymorphic expression of its two major transcripts during embryonic development. Results from bovine and ovine samples demonstrated that procollagen III mRNA expression was not detectable by the method used until day 20 of pregnancy. Hence, the major induction of this gene does not occur coincident with trophoblast elongation as observed for interferon tau (IFNτ) (Godkin, 1982; Farin, 1989 and 1990) and retinol binding protein (Liu, 1992). However expression is temporally associated with growth and development of the allantois. Production of the C-propeptide of collagen III, as observed by 2D-PAGE and fluorography, coincided with the gene expression. Since the cleavage of C-propeptide of collagen III represents the formation of the mature collagen III molecule, then onset of expression of procollagen III mRNA paralleled by synthesis and secretion of its C-terminal propeptide by chorioallantoic membranes suggests that regulation of procollagen III production may be under transcriptional control.

Coincidental gene expression of procollagen III with the development of bovine and ovine allantoic extraembryonic

membranes inferred a important role of collagen III participation in the early embryo development. Previous study of tissue distribution of different types of collagen showed type III collagen is located in blood vessels, uterus, fetal skin and placenta (Eyre, 1980; Bornstein, 1980). During early bovine and ovine embryo development, the allantois forms as an outgrowth of the hindgut. Carrying the placental vascular system, it fills and progressively fuses with the chorion between day 20 and 28 forming the chorioalltois placenta (Greenstein and Foley, 1958). After fusion, chorionic epithelium (trophectoderm) and allantoic endoderm are separated by stroma, containing few cells and extensive extracellular matrix (Doré , 1995). A previous study (chapter 3) demonstrated that allantois was the major source of C-propeptide of collagen III. We suggest that type III collagen is a major component of the choriallantois extracellular matrix and may also play an important role in embryonic angiogenesis. It is thought that active gene expression is involved in this angiogenetic period. A recent study (Doré et al., 1995) demonstrated that elevated transforming growth factor (TGF) β1 and β2 mRNA expression occured during the time of allantoic growth. Also at this time period, TGF β1 and TGF β2 proteins were identified in the allantois and placental vasculature (Doré, 1995). An earlier report showed that TGF βs strongly stimulated *in vivo* fibrosis and angiogenesis (Roberts, 1986). The β type transforming growth factors have

also been shown to have dramatic effects on several types of collagen matrix production and cell proliferation *in vitro* and *in vivo* (Rizzino, 1988; Penttinen, 1988). Study of cultured normal human fibroblast lines indicated that TGF β caused a several fold stimulation of the production of type I and type III collagens. This effect appeared to be mediated largely by a concomitant increase in the steady-state amounts of specific transcripts for these proteins (Varga, 1987). Therefore, we propose that coincidental procollagen III mRNA and protein expression with the development of chorioallantois might be regulated by peak gene expression of TGF βs during this development period.

B. Polymorphic mRNAs and utilization of multiple polyadenylation sites

Collagen gene expression studies have shown that members of this gene family transcribe more than one mRNA species. Northern blot hybridization of α1(III) and α2(V) cDNA clones to human fibroblast poly (A) RNA identified 4.9- and 5.5-kb α1(III), 5.0- and 6.3-kb α2(V) procollagen gene transcripts (Loidl, 1984; Myers, 1985). In the present study, Northern blot analysis identified two major procollagen III transcripts, 4.9 and 5.9 kb in length, in RNA samples prepared from both bovine and ovine chorioallantois (fig. 2 and 3). Similar observations of multiple transcripts were also seen in α1(I), α2(I), and α1(IV) gene (Chu, 1985; Aho, 1983; Myers,

1983; 1986). Presence of polymorphic mRNAs due to either length heterogeneity or alternative splicing has been described in several other eukaryotic genes (Tosi, 1981; Setzer, 1982; Unterman, 1981; Heilig, 1980; Bennetzen, 1982). In the collagen family, the polymorphic mRNAs seemed to be generated from alternative utilization of polyadenylation sites in the 3' untranslated region. Using our specially designed probe B, made at a region between poly (A) sites 2 and 3 from 3'-UTR of clone 9.22, only the larger transcript, 5.9 kb, was shown on Northern blots of both bovine and ovine tissues. Since poly (A) sites 1 and 2 overlapped, and the distance between poly (A) sites 3, 4, and 5 was only 97 bp and 6 bp respectively, Northern blot analysis may not have been sensitive enough to distinguish these differences and demonstrate possible additional usage of poly (A) sites. However, bovine and ovine chorion and allantois appear to utilize at least two of the poly (A) sites. This data strongly suggest the different size of bovine and ovine procollagen mRNAs are primarily due to the usage of multiple poly (A) sites.

C. Differential usage of multiple poly (A) sites

An interesting feature of procollagen III gene expression in bovine and ovine chorioallantois is the finding that expression of the two major mRNAs was developmentally regulated. In adult tissues, the type III transcripts are

about equally expressed (Myers and Dion 1990). In avian studies of α2(I) gene expression, two RNA species, 5.1- and 5.7 kb, were observed to behave differently during embryo development; the smaller species was more prominent than the larger species before day 15 of development (Merlino, 1983). Our study demonstrated that the lower molecular weight α1 (III) mRNA clearly predominated over the higher one through all the stages studied, which indicated preferential usage of the upstream poly (A) site 1 or 2 in the early stage of development. The functional importance of differential usage of the multiple poly (A) sites within the 3'-UTR of many collagen genes is still a subject of speculation. It appears, however, that usage of AATAAA hexanucleotide is not random since there is evidence suggesting a tissue-specific utilization of these conserved sequences (Barsh, 1984; Myers, 1986).

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VITA

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