Ubiquitous Expression of the α1(XIX) Collagen Gene (Col19a1) during Mouse Embryogenesis Becomes Restricted to a Few Tissues in the Adult Organism*

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Type XIX collagen is a poorly characterized member of the fibril-associated collagens with an interrupted triple helices (FACIT) class of collagen molecules. As a first step toward elucidating its function, we have isolated full size cDNA clones from the mouse α1(XIX) collagen gene (Col19a1) and established its pattern of expression in the developing embryo and adult organism. Col19a1 transcripts can be detected as early as 11 days of gestation and in all embryonic tissues, except the liver, of an 18-day postcoitum mouse. In contrast, only a few adult tissues, brain, eye, and testis, seem to accumulate Col19a1 mRNA. Col19a1 transcripts are at least 10 times more abundant in adult than fetal brain and significantly less in adult than fetal muscle and skin. Consistent with the RNA data, polyclonal antibodies for α1(XIX) collagen reacted with a 150-kDa protein in the neutral salt extraction of adult mouse brain tissues. We therefore propose that type XIX collagen plays a distinct role from the other FACIT molecules, particularly in the assembly of embryonic matrices and in the maintenance of specific adult tissues.

Collagenous networks play a critical role in the morphogenesis of the embryo and in the maintenance of tissue architecture of adult tissues. The 19 genetically distinct types of collagen molecules are expressed in developmental and tissue-specific manners (1–4). The collagen superfamily can be classified into several subgroups according to the structural features of its members, the supramolecular aggregates that they form, and the structure of the gene that the proteins are encoded. One of the most interesting and less characterized collagen subgroups is the one of the so-called FACIT (fibril-associated collagens with interrupted triple helices) which includes types IX, XII, XIV, XVI, and XIX collagen. Available evidence suggests that FACIT may play an important role in providing tissue-specific molecular links between collagen fibrils and other extracellular matrix aggregates (5, 6). With the exception of types IX and XII, virtually nothing is known about the expression pattern of the other FACITs. Without this information, no function has yet been postulated for types XIV, XVI, and XIX collagen.

Type XIX collagen was originally discovered through cDNA cloning of RNA transcripts from the human rhabdomyosarcoma cell line RD (CCL 136) (7). The predicted polypeptide was found to contain 1,142 amino acid residues with a 23-residue signal peptide followed by the five collagenous (COL) domains, interspersed and flanked with the six non-collagenous (NC) domains (3, 4, 8). The coding region of the α1(XIX) mRNA is small compared with the length of the entire transcript (10.4 kb), due to the presence of more than 5-kb long 3′-untranslated region. Additionally, an unusual number of splicing events appeared to occur in the RD cell line. More interestingly, the α1(XIX) collagen gene (COL19A1) was located to human chromosome 6q12-q14, syntenic to the α1(I) collagen (COL1A1) and α1(XII) collagen genes (COL12A1) (7, 9). Type IX collagen is found in tissues containing type II collagen, such as hyaline cartilage and the vitreous body of the eye. On the other hand, type XII collagen is found in dense connective tissues, such as tendons and ligaments, where type I is the major collagenous component. Aside from the rhabdomyosarcoma cell line, there is currently no information about the tissue distribution of type XIX collagen and, consequently, about its possible function.

The present study was designed to fill this gap of knowledge and to generate suitable reagents to eventually establish type XIX collagen function using the gene targeting approach in mouse embryonic stem cells. Accordingly, we isolated overlapping cDNA clones coding for the entire mouse α1(XIX) chain; we examined the pattern of mRNA accumulation in the tissues of both the developing and adult mice; and we identified the α1(XIX) protein in adult brain tissues.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones—Poly(A)* RNA was isolated from 18 d.p.c. mouse whole embryo tissue according to the standard protocol (10) and was used as a template for cDNA synthesis. Double-stranded cDNA was synthesized using random primers and then inserted into the agt10 vector using a commercial kit (Amersham Corp.). Three distinct mouse embryonic cDNA libraries were screened under low stringency conditions using probes specific for the human α1(XIX) collagen (4, 7). Hybridizations carried out at 55 °C overnight in a mixture containing 5 × SSC (1 × SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8), 1% N-laurylsarcosine, 50 μg/ml salmon sperm DNA, and 32P-labeled probes after prehybridization which is the same solution without probes for 1 h. Isolation and purification of the positive clones were as described previously (7). Sequencing was carried out according to the dideoxy chain termination methods using an ABI373S automatic sequencer. After sequencing validation of positive clones, a 0.6-kb gap in the α1(XIX) coding sequence was resolved by amplifying an aliquot of reverse-transcribed embryonic cDNA using the polymerase chain technique (RT-PCR). Twenty microinolites of reverse transcrip-
### Mouse α1(XIX) Collagen Gene Expression

#### Fig. 1. The domain structure of the mouse α1(XIX) collagen chain deduced from nucleotide sequences of cDNA clones and their partial restriction map.

Five overlapping cDNAs, ME1, ME2, ME12, ME14, and ME23, were isolated from 18 d.p.c. mouse whole embryo library. A clone, ME PCR, was generated from RNA of 18 d.p.c. mouse whole embryo using RT-PCR method. The alignment of these cDNAs was determined by restriction enzyme digestions and DNA sequencing. Amino acid sequence deduced from the cDNA sequence allowed the prediction of the domain structure of the mouse α1(XIX) chain (top of the figure). Numbers of the amino acid residues in individual domains of COL1–5 and NC1–6 are shown in parentheses. Locations of the 16 cysteinyl residues are indicated by closed circles. Positions of the nine imperfections of the Gly-X-Y repeated structure are shown by short bars above the COL domains. The signal peptide of 23 amino acid residues is indicated by a thicker bar at the amino terminus of the chain. Locations of 5’ and 3’ mouse probes used for the Northern blotting analysis (in Fig. 6) are indicated by thin bars with the numbers 1 and 2 in parentheses.

| Restriction Enzyme | ME1 (1kb) | ME2 (0.5kb) | ME12 (1.2kb) | ME23 (0.6kb) | ME14 (2.3kb) | ME PCR (0.8kb) |
|-------------------|-----------|------------|-------------|-------------|-------------|---------------|
| EcoRI             |           |            |             |             |             |               |
| HindIII           |           |            |             |             |             |               |
| PstI              |           |            |             |             |             |               |
| SmaI              |           |            |             |             |             |               |
| SacI              |           |            |             |             |             |               |
| XhoI              |           |            |             |             |             |               |
| SalI              |           |            |             |             |             |               |

#### Northern Blot and RT-PCR Analysis

- **Preparation of Type XIX Collagen Antibodies**—A recombinant α1(XIX) NC1-COL1-NC2 peptide was made by expressing a cDNA fragment subcloned into EcoRI and PstI sites of pMALc2 (New England BioLabs Inc.) expression plasmid. Production of the MBP (maltose binding protein)-α1(XIX)COL fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration of 0.3 mM) to the bacterial cultures incubated at 37 °C for 2 h. The cells were lysed by lysozyme treatment, and the fusion proteins were purified by affinity chromatography on an amylose column as described by the manufacturer (New England Biolabs Inc.). After incubation of the fusion protein with Xa-specific peptidease, the α1(XIX)COL recombinant protein was separated on 12.5% SDS-PAGE gel and isolated in a gel slice after Coomassie Brilliant Blue R (Merck, Darmstadt, Germany) staining (12). The gel slices containing approximately 500 μg of protein in total were washed with phosphate-buffered saline, cut into the small pieces, emulsified with the equal volume of complete Freund’s adjuvant (Difco), and then injected subcutaneously into a rabbit. Booster injections (200 μg) were given with incomplete Freund’s adjuvant (Difco) 3
Mouse a1(XIX) Collagen Gene Expression

Fig. 2

NC 6

COL 5

NC 5
weeks after the first injection. The titers of antisera were measured with enzyme-linked immunosorbent assay (13).

Collagen Purification—All steps were carried out at 4 °C or on ice (14). Brains, dissected from 40 5-week-old mice, were homogenized in 500 ml of cold isotonic buffer (0.25M sucrose, 10 mM HEPES, pH 7.0, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide), and then stirred for 60 min. The homogenate was centrifuged for 30 min at 15,000 rpm. The pellet was suspended in 500 ml of extraction buffer (1 M NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide) and incubated for 48 h with stirring. The suspension was centrifuged at 35,000 rpm for 30 min, and the solubilized proteins were precipitated with 4M NaCl of final concentration. The precipitate was collected by centrifugation at 25,000 rpm for 30 min, and then the pellet was suspended in 0.5M acetic acid.

**Fig. 2**—continued

![Diagram](Image)

**FIG. 2.** Nucleotide sequence of cDNA (top) and deduced amino acid sequence (middle) of the mouse α1(XIX) collagen chain. The amino acid sequence of the human (bottom) has been aligned with that of the mouse chain. The residues of the human differing from those of the mouse only are shown. The collagenous domains (COL) are indicated by boxes, and those of the imperfections are indicated by small boxes in them. The asterisks, the triangle, and open squares indicate the amino acid residues missing in the mouse and a stop codon, the putative signal peptide cleavage site, and cysteine residues. The horizontal arrows show the positions of the primers; primers 1–4 were used for generating cDNA clone, ME PCR, primer 5 and 6 for RT-PCR analysis, and primer 7 and 8 for generating DNA fragment to be ligated to expression vector.
the gel. The sample for digestion was first neutralized with 0.5M NaOH and then incubated with bacterial collagenase solution (250 units/ml) containing 5.5 mM CaCl₂ for 5 h at 37°C. Samples were transferred onto polyvinylidene difluoride membranes electrophoretically and probed with antibody against anti-rabbit IgG. The signals were detected by enhanced chemiluminescence Western blotting detection reagents (Amersham Corp.).

The symbols used are: ++, strongly positive; +, weakly positive; ±, trace; and −, negative.

|            | 18 d.p.c. embryo | 5-Week-old adult |
|------------|-----------------|-----------------|
| Muscle     | +               | −               |
| Heart      | +               | −               |
| Aorta      | ND              | ±               |
| Skin       | +               | −               |
| Intestine  | −               | −               |
| Brain      | +               | +               |
| Whole      | +               | +               |
| Cerebrum   | +               | +               |
| Cerebellum | ±               | ±               |
| Eye        | ND              | −               |
| Limb       | ND              | ND              |
| Vertebrae  | +               | ND              |
| Tail       | −               | −               |
| Joint      | ND              | −               |
| Sternum    | ND              | ND              |
| Calvaria   | +               | ND              |
| Lung       | +               | ±               |
| Kidney     | +               | ±               |
| Liver      | −               | −               |
| Spleen     | ND              | −               |
| Thyroid    | ND              | −               |
| Adrenal    | ND              | −               |
| Testis     | ND              | +               |

The transcripts are usually not detectable in this condition. Compare to the results of competitive RT-PCR in Table III.

Table II

| Expression of α1(IX) collagen mRNAs in the cultured cells determined by RT-PCR analysis |
|----------------------------------------------------------------------------------------|
| The symbols used are: ++, strongly positive; +, weakly positive; ±, trace; and −, negative. |

| Human Tumor (Rhabdomyosarcoma) | + |
|---------------------------------|---|
| Human 293 (Glioma)             | + |
| Mouse Neuroblastoma (129F1)     | + |
| Mouse Neuroblastoma (A172)      | + |
| Mouse Fibroblast (M13)          | ± |
| Mouse Embryo (129F1)            | + |
| Mouse Embryo (A172)             | + |
| Mouse Embryo (M13)              | ± |
| Mouse Embryo (EB virus)         | − |

The symbols used are: ++, strongly positive; +, weakly positive; ±, trace; and −, negative.

Results and Discussion

Primary Structure of Mouse α1(IX) Chain—To identify the best source for α1(IX) collagen, we initially performed RT-PCR amplifications using RNA from a variety of embryonic and adult mouse tissues and a couple of sets of primers derived from the human sequence. The most convincing results of this initial survey were obtained using embryonic tissues. Sequencing of these amplification products showed that the clones were the mouse counterparts of the human COL19A1 gene. Based on these data, we constructed and screened a cDNA library from 18 d.p.c. mouse whole embryo. Five overlapping clones were isolated and, upon sequencing, found to cover all but 0.6 kb of the endogenous product (closed arrowhead). On each gel, lanes left to right contain amplified DNA synthesized from a constant amount of tissue cDNA and decreasing amounts of competitor DNAs. The DNA competitor in the reaction mix was as follows: α1(IX) collagen competitor: lane A, 1 pg; lane B, 500 fg; lane C, 250 fg; lane D, 125 fg; lane E, 62.5 fg; lane F, 31.3 fg; lane G, 15.6 fg; lane H, 7.8 fg; lane I, 3.9 fg; lane J, 1.9 fg; lane K, 1 fg; and β actin competitor: lane 1, 50 pg; lane 2, 25 pg; lane 3, 12.5 pg; lane 4, 6.3 pg; lane 5, 3.2 pg; and lane 6, 1.6 pg.

Fig. 3. Competitive PCR products run on the agarose gels. The gel shown here contains RT-PCR products of one representative experiment using the muscle, skin, brain, and liver RNAs as templates. The concentration of the gels is 2.0% for α1(IX) and 2.5% for β actin. The products from RNA of β actin are shown only from the experiment using the skin. The panels on the left show the products from RNAs of 18 d.p.c. mouse embryo, and on the right from 5-week-adult mouse. The RT-PCR products of the competitor (open arrowhead) has been designed to be 51 bp (IXIX collagen) and 61 bp (β actin) smaller than those of the endogenous product (closed arrowhead). On each gel, lanes left to right contain amplified DNA synthesized from a constant amount of tissue cDNA and decreasing amounts of competitor DNAs. The DNA competitor in the reaction mix was as follows: α1(IX) collagen competitor: lane A, 1 pg; lane B, 500 fg; lane C, 250 fg; lane D, 125 fg; lane E, 62.5 fg; lane F, 31.3 fg; lane G, 15.6 fg; lane H, 7.8 fg; lane I, 3.9 fg; lane J, 1.9 fg; and lane K, 1 fg; and β actin competitor: lane 1, 50 pg; lane 2, 25 pg; lane 3, 12.5 pg; lane 4, 6.3 pg; lane 5, 3.2 pg; and lane 6, 1.6 pg.

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The deduced amino acid sequence of the mouse α1(IX) chain shows 82% identity to the human counterpart with the highest level of sequence conservation at the carboxyl termini than the amino termini. All of the potentially important structural-functional features previously noted in the human α1(IX) chain are also conserved in the mouse polypeptide (Fig. 2). The major difference between the two mammalian polypeptides is the total number of 1,136 amino acids in mouse versus 1,142 amino acids in human. The differences are due to the deletion of three amino acids in each NC6 and NC5 domain and the substitution of cysteinyI residues for serinyI residues in the signal peptide of the mouse chain. Additional structural differences include the NC6 and NC3 domains, which are shorter in the mouse compared with the human chain, and the relative
position of small imperfection in the COL5 domain. These few differences notwithstanding, the primary structure of the α1(XIX) collagen chains is remarkably conserved between the two mammalian species.

Tissue Distribution of Col19a1—To define the pattern of expression of Col19a1, we surveyed tissues from various embryonic stages and 5-week-old mouse tissues. The transcripts were readily detectable by RT-PCR analysis in the whole embryo at 11, 12, and 14 d.p.c. (data not shown). Positive tissues of 18 d.p.c. embryo include limbs, vertebrae, heart, brain, tail, kidneys, calvaria, lung, muscle, skin, and intestine (Table I). A very different pattern was observed in adult tissues where Col19a1 expression was only seen in the cerebrum, cerebellum, eyes, and testis, and perhaps in the aorta, lungs, and kidneys (Table I). Based on these data, we used the same approach to identify cultured cells that accumulate COL19A1 (and Col19a1) transcripts. This second survey included human rhabdomyosarcoma cell lines (RD, A204, and KMY1), human glial cell lines (U251MG, U373MG, and A172), human neuroblastoma cell lines (TGW and NB1), human normal skin fibroblasts (infant and adult), and mouse normal skin fibroblasts (18 d.p.c. embryo and adult) (Table II). Among malignant cell lines, only RD (rhabdomyosarcoma) and U251MG (glioma) showed significant COL19A1 mRNA accumulation. Detectable amounts were also noted in human neonatal fibroblasts, mouse embryonic tissues, and to a much lesser extent, in adult fibroblasts.

To examine whether Col19a1 gene expression may change during development, we compared transcript levels in the muscle, skin, brain, and liver of 18 d.p.c. embryos and 5-week-old mice using the quantitative technique of competitive RT-PCR (Fig. 3). Relative quantitation of amplification from the endogenous transcript and the competitor plasmid revealed that 1 μl of cDNA from muscle, skin, brain, and liver of 18 d.p.c. embryo and 5-week-old mouse contained 52.3, 7.1, 24.5, 9.7, and 149.3 fg, respectively. When normalized to the β-actin values, the relative amounts of Col19a1 transcripts were significantly higher in embryonic muscle and skin than in the adult counterparts, whereas transcript levels in the brain were approximately 10 times more in adult animals than embryos (Table III).

To identify the protein in a tissue that expresses the α1(XIX) mRNA, we utilized specific polyclonal antibodies (Fig. 4) in a Western blotting analysis of mouse adult brain tissue. The antibodies were raised in rabbits against an α1(XIX) recombinant peptide. As shown Fig. 5C, the antibodies reacted with peptides of 150 and 145 kDa in size from a fraction of neutral salt extraction of mouse adult brain. Consistent with their identity, the 150- and 145-kDa bands were digested with bacterial collagenase (Fig. 5C, lane 2). Under nonreducing conditions, the reacting peptide migrated at about 400 kDa which probably represents the trimeric form of α1(XIX) collagen.

The above results suggest that type XIX collagen is an embryonic FACIT, since its expression is mostly confined to embryonic tissues and only a few adult organ systems. High Col19a1 expression in a couple of tumor lines indirectly supports the idea of a mostly embryonic gene product. We postulate that the uniqueness of this FACIT molecule extends to the kind of supermolecular aggregate it is associated with. We note that Col19a1 expression in the embryo does not follow the

| Table III | Mean ratios of mouse α1(XIX) collagen to β actin mRNA (×10⁻³) |
|-------|--------------------------------------------------|
|        | 18 d.p.c. embryo | 5-Week adult |
| Muscle | 6.03 ± 4.13 | 0.14 ± 0.10 |
| Skin  | 2.00 ± 1.20 | 0.15 ± 0.19 |
| Brain | 1.15 ± 0.47 | 13.57 ± 8.08 |
| Liver | ND           | ND           |

FIG. 4. Enzyme-linked immunosorbent assay using anti-type XIX collagen polyclonal antibody. The antibody reacted with the α1(XIX)-MBP fusion protein (closed circles) but never cross-reacted with total Escherichia coli proteins containing MBP (open squares).

FIG. 5. Protein analysis for the type XIX collagen using mouse adult brain extract. A, purified recombinant proteins were run on 12.5% SDS-PAGE gel under a reducing condition and stained with Coomassie Brilliant Blue. Lane 1, MBP-COL fusion protein purified with amylose affinity column; lane 2, digested fusion protein with factor Xa; lane 3, digested protein with bacterial collagenase. An arrow indicates COL peptide in lane 2, which disappears in lane 3. B, gel electrophoresis of the extract prepared from mouse brain. The sample was separated on 6.0% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 1, neutral 1 M NaCl extract from mouse adult brain tissues; lane 2, the same sample of lane 1 treated with bacterial collagenase; lane 3, the same sample of lane 1 under nonreducing condition; lane 4, mouse type I collagen; lane 5, mouse type I collagen digested with bacterial collagenase. C, Western blot analysis for neutral 1 M NaCl extract prepared from mouse adult brain tissues. Lanes 1-3 correspond with lanes 1-3 of B, respectively. Molecular mass is indicated on the left side of the panels.
Alternative splicing of the COL19A1 transcript—We have previously reported an unusual number of alternatively spliced COL19A1 products in the rhabdomyosarcoma cell line RD (4). Here we examined whether or not alternatively spliced products appear to be tissue- and/or stage-specific. Northern analysis of mRNA from 18 d.p.c. whole embryo yielded a single band with 5′ (Fig. 6A, lane 3) and 3′ (lane 4) cDNA fragment as probes. Transcripts from mouse adult brain also showed a single band with more strong signal (Fig. 6B, lane 3) than that from 18 d.p.c. mouse embryo (Fig. 6B, lane 2) when 5′ probe was used. Likewise, mRNA from infant skin fibroblasts showed only one hybridizing species (Fig. 6C, lane 3). In contrast, mRNA from glioma cell line (U251MG) yielded multiple bands (Fig. 6C, lane 2).

Alternative splicing is a widely used means to generate protein isoforms or even functionally distinct products from the same transcript. Alternative splicing has been reported to occur for several different collagen types (17–24), although its functional significance is understood only for a few of them. In the case of the COL19A1 transcripts seen in the tumor cells are probably aberrant products of no physiological significance and without counterparts in the normal organism. It is however unclear the reason for and the mechanism behind this strange phenomenon in cancer cells.

Conclusion—In summary, this study has provided new and important information about the possible role of type XIX collagen. We believe the expression data are consistent with the idea that type XIX collagen is somehow involved in the assembly of specialized structures of the embryonic and/or the function of particular organ systems in the adult organism. Irrespective of the hypothesis, it is safe to predict that type XIX collagen will eventually prove to be unique among other FACIT members. The information and reagents produced by the present study provide the means to address this question using the powerful technique of gene targeting in the mouse.

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Mouse α1(XIX) Collagen Gene Expression

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