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Leena Ala-Kokko
Thomas Jefferson University

James Hyland
Thomas Jefferson University

Carol Smith
Thomas Jefferson University

Kari I. Kivirikko
University of Oulu

Sergio A. Jimenez
Thomas Jefferson University, Sergio.Jimenez@jefferson.edu

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Authors

Leena Ala-Kokko, James Hyland, Carol Smith, Kari I. Kivirikko, Sergio A. Jimenez, and Darwin J. Prockop

Communication

Expression of a Human Cartilage Procollagen Gene (COL2A1) in Mouse 3T3 Cells*

Leena Ala-Kokko‡, James Hyland‡, Carol Smith‡, Kari I. Kivirikko§, Sergio A. Jimenez‡¶, and Darwin J. Prockop‡¶‖

From the ‡Department of Biochemistry and Molecular Biology and ¶Department of Medicine, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the §Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, SF-90220 Oulu, Finland

Expression in a recombinant system has been difficult to obtain for any of the major fibrillar collagens that require processing by eight or more post-translational enzymes. Here, two DNA constructs were designed so that the promoter region of the gene for the pro-α1(I) chain of human type I procollagen drove expression of the human type II procollagen gene in mouse NIH 3T3 cells, a culture line that normally synthesizes type I procollagen but not any cartilage-specific protein such as type II procollagen. Both constructs were expressed as both mRNA and protein. In clones expressing the construct at high levels, the steady-state levels of mRNA and the production of type II procollagen were comparable to the mRNA levels and production of type I procollagen from the endogenous mouse genes. Comparison of clones containing the two constructs demonstrated that sequences extending 80 base pairs beyond the major polyadenylation signal of the gene are not in themselves sufficient for correct termination and 3′ processing of RNA transcripts. The results strongly suggest that specific sequences present in a downstream 3.5-kilobase SphI/SphI fragment determine the termination of the transcription. Of special importance is that the system will make it possible to examine the consequences of mutations in the human type II procollagen gene on the processing of RNA transcripts and on the functional properties of the protein simply by using the genomic DNA from leukocytes or other non-cartilaginous sources.

Expression of many exogenous genes is readily obtained in a variety of recombinant host-vector systems. Expression of an exogenous gene, however, is difficult to obtain if the protein normally requires extensive post-translational processing. Apparently for this reason, expression in a recombinant system

has not been reported for any of the major fibrillar collagens that require processing by eight or more post-translational enzymes (1). Rescue experiments in cells that synthesized only one of the two chains for type I procollagen were successful in two different systems (2, 3), but synthesis of a procollagen molecule in which all three chains are derived from an exogenous gene has not been obtained. Failure to obtain expression of genes for fibrillar collagens in a fully recombinant system has hampered attempts to study the normal structure-function relationships of the proteins and to study the effects of mutations. In particular, mutations in the gene for type II procollagen have recently been implicated as the cause of several human diseases (4–13), but because adequate numbers of human cartilage cells are difficult to obtain and because human chondrocytes readily lose their phenotype in culture (14, 15), the causal relationship between a mutation in the gene and the biological function of the protein has proven elusive. Here we describe a system in which it is possible to express the human type II procollagen gene in stable transfectants of mouse NIH 3T3 cells. The results also indicate that correct termination and 3′ processing of RNA transcripts of the gene require sequences over 80 bp[1] downstream of the major polyadenylation signal (16).

MATERIALS AND METHODS

Gene Constructs—Two versions of a cosmid construct were prepared. Both contained a 5′-fragment from the human COL1A1 gene that included the promoter, the first exon, and most of the first intron (Fig. 1). The 5′-fragment extended from −500 to +1445 bp of the gene (17–19). Both constructs also contained two SphI/SphI fragments of 14 and 12 kb of the human COL2A1 gene (20–27). The 5′-end of the 14-kb fragment corresponded to the 3′-end of intron 1B (28), and the 3′-end of the 12-kb fragment extended 80 bp beyond the major polyadenylation signal of the gene. One construct differed from the other in that it contained an additional 3.5-kb SphI/SphI fragment from the 3′-end of the gene.

The first construct was assembled from three fragments: (*a*) a 2-kb SphI/HindIII fragment from the 5′-end of human COL1A1 gene in which the SphI site was converted to a SalI site and the HindIII site was converted to an SphI site; (*b*) a 14-kb SphI/SphI fragment from the middle of the human COL2A1 gene; and (*c*) a 12-kb SphI/SphI fragment that extended 80 bp beyond the major polyadenylation signal of the human COL2A1 gene and in which the 3′-SphI site was converted to a SalI site. The three fragments were assembled by four-way ligation into the SalI site of the cosmid vector pJB8 that was previously modified by insertion of a 7-kb EcoRI/EcoRI stuffer fragment (27).

The second construct was assembled in two steps. The first step involved (*a*) the 5′-fragment from the COL1A1 gene with SalI/SphI terminal sites used for the first construct and (*b*) a 3.5-kb SphI/SphI fragment that extended beyond the 3′-end of the COL2A1 gene. The 3′-SphI site in the 3.5-kb SphI/SphI fragment was converted to a SalI site. In the first step, the 2-kb SalI/SphI and 3.5-kb SphI/SalI fragments were assembled into the modified cosmid vector by three-way ligation. In the second step the two SphI/SphI fragments of 14 and 12 kb from the COL2A1 gene were inserted by three-way ligation into the SphI site of the construct obtained in the first step.

Cell Transfections—For the cell transfection experiments, a cosmid clone containing a chimeric COL1A1/COL2A1 gene was cleaved with SalI. A plasmid containing a neomycin-resistent gene (29) was linearized by cleavage with BamHI. The two samples were mixed in a

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[1] The abbreviations used are: bp, base pair(s); kb, kilobase(s); COL1A1, gene for the pro-α1(I) chain of type I procollagen; COL2A1, gene for the pro-α1(II) chain of type II procollagen; SDS, sodium dodecyl sulfate.

ratio of 10:1 of chimeric gene construct to neomycin-resistant gene, and the mixture was then used for co-transfection of NIH 3T3 cells by calcium phosphate precipitation (30). The DNA in the calcium phosphate solution was layered onto cultured cells with about 10 μg of chimeric gene construct per 90-mm plate of preconfluent cells. The cells were incubated in Dulbecco's modified Eagle's medium containing 10% newborn calf serum for 10 h. The samples were subjected to glycerol shock by adding a 15% glycerol solution for 3 min. The cells were transferred to Dulbecco's modified Eagle's medium containing 10% newborn calf serum for 24 h and then to the same medium containing 450 μg/ml G418. Incubation in the medium containing G418 was continued for about 4 weeks with a change of medium every third day. The G418-resistant cells were either pooled or used to obtain clones by isolating foci with a plastic cylinder and subculturing.

Northern Blot and Western Blot Analysis—Total RNA was extracted from stably transfected NIH 3T3 cells with guanidinium isothiocyanate and the RNA purified by centrifugation in cesium chloride (30). The RNA samples were separated by agarose gel electrophoresis and blotted onto nitrocellulose filters for hybridization with human cDNAs for COL2A1 (31) and COL1A1 (32).

For Western blot analysis, the culture medium from each of several clones was removed and separately precipitated by the addition of solid ammonium sulfate (30% saturation). The precipitates were collected by centrifugation at $14,000 \times g$ and then dialyzed against a buffer containing 0.15 M NaCl, 0.5 mM EDTA, 0.5 mM N-ethylmaleimide, 0.1 mM p-aminobenzamidine, and 50 mM Tris-HCl (pH 7.4 at 4 °C). Aliquots of the samples were heated to 100 °C for 5 min in 1% SDS, 50 mM dithiothreitol, and 10% (v/v) glycerol, and separated by electrophoresis on 6% polyacrylamide gels on a mini-gel apparatus (Holford SE250, Holford Scientific) at 125 V for 90 min. The separated proteins were electroblotted at 40 V for 90 min from the polyacrylamide gel onto a supported nitrocellulose membrane (Schleicher and Schuell). The transferred proteins were reacted for 30 min with a 1:500 dilution (v/v) of a polyclonal antibody specific for the COOH-terminal telopeptide of human type II collagen. The antibody was prepared in rabbits with a 23-residue synthetic peptide that had an amino acid sequence found in the COOH-terminal telopeptide of human type II collagen (22). The antibody did not react by Western blot analysis with chains of human type I procollagen, human type I collagen, human type III procollagen, or murine type I procollagen. The proteins on the filter reacting with the antibody were detected with a secondary anti-rabbit IgG antibody coupled to alkaline phosphatase (Promega Biotec) for 30 min. The alkaline phosphatase was visualized with NBT/BCIP (Promega Biotec) as directed by the manufacturer.

Cyanogen Bromide Peptide Analysis of Type II Collagen—Pooled samples of medium and cell layer of clones expressing the human COL2A1 gene were digested at 15 °C with 100 μg/ml pepsin at pH 2.0 for 5 h, and the proteins were precipitated from the acidic solution with 0.8 M NaCl. The pellets were collected by centrifugation at $14,000 \times g$ for 30 min and resuspended in 0.15 M NaCl in 50 M Tris-HCl buffer, pH 7.4, at 4 °C. The proteins were then separated by polyacrylamide gel electrophoresis in SDS and 6% polyacrylamide gels as described above. The gels were equilibrated with 70% (v/v) formic acid for 10 min twice and digested with excess CNBr for 90 min at 25 °C. The gels were placed perpendicularly to the direction of electrophoresis in the first dimension and electrophoresed in the second dimension on polyacrylamide gels (6% stacking gel and 12% resolving gel) for 90 min. The separated peptides were electroblotted onto nitrocellulose membranes for 60 min as described above. The peptides were visualized by reaction with rabbit polyclonal antibodies that recognized multiple epitopes in human type II collagen. The antibodies were generously provided by Dr. Daniel Hartmann (Pasteur Institute, Lyon, France). The secondary antibody was anti-rabbit IgG coupled to alkaline phosphatase (Promega Biotec).

RESULTS AND DISCUSSION

The two gene constructs employed here (Fig. 1) were designed to test the hypothesis that the promoter of the human COL1A1 gene would drive expression of human COL2A1 gene in mouse NIH 3T3 cells that normally express type I procollagen but do not synthesize any cartilage-specific proteins such as type II procollagen. The constructs were used to generate stably transfected cells, and the cells were assayed for synthesis of mRNA for human type II procollagen (Fig. 2). Cells transfected with either construct synthesized human
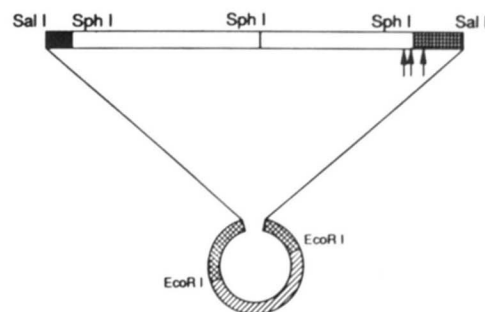

FIG. 1. **DNA constructs for expression of the human type II procollagen gene.** The 7-kb *Eco*RI stuffer was from the human type II procollagen gene (22, 27). The *three arrows* indicate the three polyadenylation signals AATAAA, ATTAAA (the major signal in the COL2A1 gene), and AATAAA, respectively. As indicated in the text, two constructs were prepared. One contained the 3.5-kb 3'-*Sph*I/*Sal*I fragment, and the other lacked the 3.5-kb fragment. ■, promoter, exon 1, and intron 1 of COL1A1 gene; □, intron 1B to exon 52 of COL2A1 gene; ■, 3.5-kb 3'-end of COL2A1 gene; ■, pJB8 cosmid vector; ▨, 7-kb *Eco*RI.
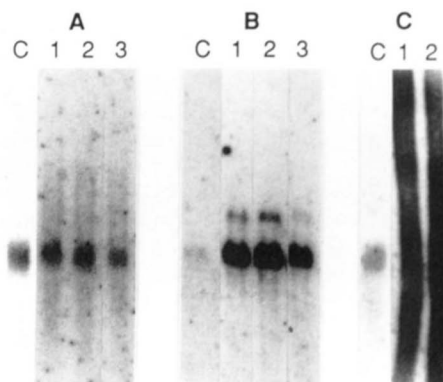

FIG. 2. **Northern blot analysis of mRNA from control and transfected cells.** *Panel A*, control RNA from human fetal chondrocytes (*C*) (31) and RNA from three cell lines transfected with the longer gene construct (Fig. 1). The filter was hybridized with a human COL2A1 cDNA (31). *Panel B*, same RNA as in *panel A* hybridized with a human COL1A1 cDNA (32). *Panel C*, control RNA from human fetal chondrocytes and RNA from two clones containing the shorter gene construct (Fig. 1). The filter was hybridized with the same human COL2A1 cDNA as in *panel A*. The major bands of mRNAs in *panels A* and *B* are about 5 and 5.8 kb.

type II procollagen mRNA. The mRNA from cells containing the construct that included the 3.5-kb *Sph*I/*Sph*I fragment extending beyond the 3'-end of the gene appeared as a discrete band of the expected size of about 5 kb (Fig. 2A). In contrast, the mRNA from cells containing the shorter construct (Fig. 2C) appeared as a broad smear ranging in size from RNAs larger than the expected message for type II procollagen to much smaller sizes. The results, therefore, demonstrated that sequences extending 80 bp beyond the unusual ATTAAA major polyadenylation signal (16) of the gene are not in themselves sufficient for correct termination and the 3'-processing of RNA transcripts that is required to generate stable mRNA. The 80-bp region contains a GT-rich sequence (16) required for correct polyadenylation of transcripts of most eukaryotic genes (33). To date, specific sequences that determine the termination of transcription in eukaryotic cells have not been defined (33). The results here strongly suggest that such specific sequences are present in the 3.5-kb *Sph*I/*Sph*I fragment of the human COL2A1 gene.

The transfected cells were then assayed for expression of the type II procollagen gene as protein by Western blot

analysis. For this purpose, a polyclonal antibody was prepared in rabbits with a 23-residue synthetic peptide that had an amino acid sequence found in the COOH-terminal telopeptide of human type II collagen (22). Western blots demonstrated the presence of human pro-α1(II) chains in the media from several stably transfected lines of 3T3 cells (Fig. 3). To verify that the transfected cell lines were synthesizing human type II procollagen, two-dimensional cyanogen bromide peptide mapping was carried out (Fig. 4). The peptides were identified by Western blotting with polyclonal antibodies that reacted with multiple epitopes in the triple helix of type II collagen. The protein generated the expected pattern of cyanogen bromide fragments for type II collagen.

The level of expression of the human COL2A1 gene as mRNA and protein varied in different lines and clones of the neomycin-resistant cells. In the highest expressing clones, the steady-state levels of mRNA for type II procollagen as assayed with a cDNA for the human protein were comparable with the endogenous levels of mRNA for mouse type I procollagen as assayed with a cDNA for the human pro-α1(I) chain. In the same clones, the production of human type II procollagen was comparable with the production of endogenous mouse type I procollagen as assayed by semi-quantitative Western blotting or by chromatographic purification of the proteins from culture media (not shown). In general, there was a good correlation between the steady-state levels of mRNA and the production of type II procollagen. Surprisingly, there were no consistent differences in levels of type II procollagen production among clones transfected with the shorter construct and cells transfected with the longer construct containing the 3.5-kb *SphI/SphI* fragment. The longer and apparently less stable transcripts from the shorter gene constructs were, therefore, efficiently translated. Clones producing high levels of type II procollagen were stable and continued to synthesize the protein even after repeated passage in culture and storage as frozen cells over a 2-year period (not shown).

The results here demonstrate that the promoter region together with the first exon and 1,218 bp of the first intron of the human COL1A1 gene are sufficient to drive expression of a cartilage-specific collagen gene in cells that do not nor-
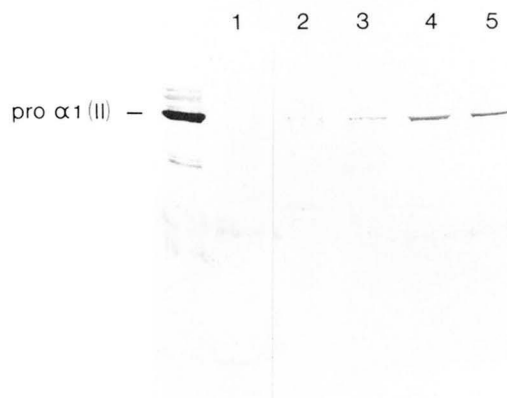

1st Dimension →

2nd Dimension

CB10,5α1(II)
CB11α1(II)
CB8α1(II)
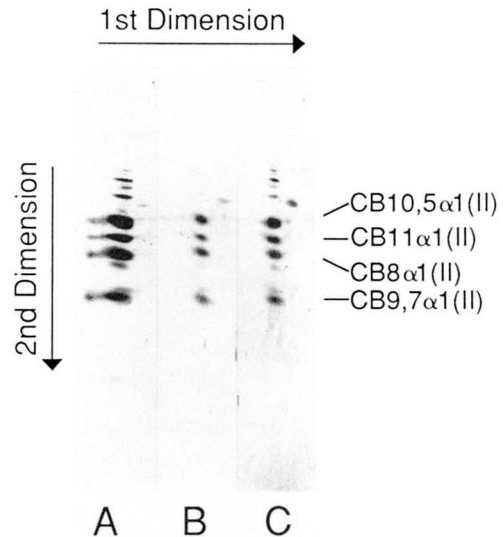CB9,7α1(II)

A   B   C

FIG. 4. **CNBr peptide analysis of type II collagen.** Pooled samples of medium and cell layer from clones expressing the human type II procollagen gene were pepsin-digested, salt-precipitated, and separated by polyacrylamide gel electrophoresis in SDS. The gels were digested with CNBr and electrophoresed in a second dimension. The peptides were electroblotted and visualized with polyclonal antibodies that reacted with multiple epitopes in the triple helix of type II collagen. For comparative purposes, only regions containing peptides from α chains are shown. *Lane A*, control sample of type II collagen from human fetal chondrocytes; *lanes B* and *C*, samples from two separate clones stably transfected with the shorter gene construct shown in Fig. 1. The control sample also contained a small amount of the 3α chain of type XI collagen.

mally express any cartilage gene. The results, therefore, are consistent with previous reports suggesting that the promoter with or without elements in the first intron is sufficient for tissue-specific expression of the COL1A1 gene (34–38).

Of special importance is that the system developed here provides a unique method for obtaining human cartilage collagen from stably transfected murine cells. Extensive efforts were made in the past to establish human chondrocytes that continue to synthesize type II procollagen, but it is difficult to expand the cultures without a loss of phenotype (14, 15). Also, it is usually difficult to obtain sufficient amounts of human articular cartilage. These technical limitations have presented serious obstacles in developing definitive data to confirm observations suggesting that mutations in the type II procollagen gene can cause several human diseases, including chondrodysplasias (5–7), arthro-ophthalmopathy (Stickler syndrome) (8–10), and primary generalized osteoarthritis (11–13). The system developed here makes it possible to generate both mRNA and protein simply by using genomic DNA from leukocytes or other non-cartilaginous sources. Therefore, it makes it possible to examine directly the effects of mutations in the human COL2A1 gene on the processing of RNA transcripts and on the functional properties of type II procollagen.

1   2   3   4   5

pro α1 (II) —

FIG. 3. **Western blot analysis of proteins synthesized by individual clones expressing the human type II procollagen gene.** Proteins from culture media were reduced and separated by polyacrylamide gel electrophoresis in SDS and were electroblotted onto nitrocellulose filters. The filters were reacted with the polyclonal rabbit antibodies to a 23-residue synthetic peptide (described in the text) that were specific for human type II procollagen and collagen. The secondary antibody was an anti-rabbit IgG linked to alkaline phosphatase. *Left lane*, type II procollagen from human fetal chondrocytes (31). *Lane 1*, control 3T3 cells that were not transfected; *lanes 2–5*, clones of 3T3 cells transfected with the shorter construct shown in Fig. 1.

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