Lack of a phenotype in transgenic mice aberrantly expressing COL2A1 mRNA because of highly selective post-transcriptional down-regulation

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We reported previously that a 1.9-kb 5'-fragment from the human *COL1A1* gene drove transcription of a promoterless human *COL2A1* gene in tissues of transgenic mice that normally express the *COL1A1* but not the *COL2A1* gene. In the present study, we have established that the aberrant transcription of the *COL2A1* gene did not produce any gross or microscopic phenotype, because the transcripts were not efficiently translated in cells that do not normally express the *COL2A1* gene. In two lines of transgenic mice, the mRNA levels from the transgene were 30% to 45% of the mRNA for the pro α 1(I) chain of type I procollagen, the most abundant mRNA in the same tissues. Analysis of collagens extracted from skin of the transgenic mice indicated that triple-helical type II collagen, with the normal

pattern of cyanogen bromide peptides, was synthesized from the transgene. However, the level of type II collagen in skin was less than 2% of the level of type I collagen. Hybridization *in situ* indicated the presence of mRNA for both *COL2A1* and *COL1A1* in the same cells. Immunofluorescence staining for type II collagen, however, was negative in the same tissues. The results, therefore, indicated that many mesenchymal cells in the transgenic mice had high steady-state levels of the homologous mRNAs for type I and type II procollagen, but only the mRNAs for type I procollagen were efficiently translated.

Key words: COL1A1 promoter, collagen, elective translation.

INTRODUCTION

Type II collagen is similar in structure to type I collagen, but differs in tissue distribution and the morphology of the fibres formed [1,2]. Type II collagen is found primarily in hyaline cartilage, where it is assembled into thin fibrils. The fibrils are organized into arcade-like structures that are distended by the presence of proteoglycans and that make the cartilage resilient to compression. Type I collagen is found in a large number of non-cartilaginous connective tissues such as bone, skin and tendon. The fibrils are thicker than the fibrils of type II collagen, and they frequently aggregated into large fibres that have impressive tensile strength.

We have described previously transgenic mice expressing a hybrid *COL1A1*–*COL2A1* construct in which a 1.9-kb 5'-fragment of the *COL1A1* gene, containing only 476 bp of the promoter, was linked to a promoterless 29.5-kb fragment of the *COL2A1* gene for human type II procollagen [3]. High levels of mRNA from the hybrid construct were present in tissues that normally synthesize type I but not type II procollagen and collagen. Also, processed pro α 1(II) chains and cross-linked α 1(II) chains of type II collagen were detected in the same tissues by a highly sensitive Western-blot assay. In the present work, we prepared two additional lines of mice expressing the *COL1A1*–*COL2A1* hybrid construct and determined whether the aberrant expression of the *COL2A1* gene produces a phenotype. The results demonstrated that no phenotype was produced, because the mRNA for type II procollagen was not efficiently translated in cells containing high levels of the homologous mRNAs for both type I and type II procollagen.

MATERIALS AND METHODS

Gene constructs and transgenic mice

The *COL1A1*–*COL2A1* hybrid construct was prepared as described previously from fragments of both genes [4]. The final construct contained 476 bp of the promoter, the first exon and most of the first intron of the *COL1A1* gene, extending up to +1445 bp. The *COL1A1* fragment was linked to 29.5 kb of sequences of the human *COL2A1* gene that extended from the 3'-end of the second intron of the gene to about 3.5 kb beyond the major polyadenylation signal of the gene. The construct was used to prepare transgenic mice in inbred FVB/N males and females [3]. Founder mice were identified by PCR assays of toe extracts and a total of four lines of mice were prepared (BSG-1, BSG-2, BSG-3 and BSG-4). The mice were propagated by breeding into the same FVB/N strain.

Quantitative reverse transcription (RT)-PCR assay for expression of the transgene

For RT, total RNA was extracted from skin or bone samples (Rapid Total RNA Isolation Kit; 5 Prime $\rightarrow$ 3 Prime Inc., Boulder, CO, U.S.A.). RT was carried out using 2.0 μ g of total RNA and the primers BS94 and BS92 [3]. For PCR amplification, three primers were used. The 5'-primer was ³²P-labelled and directed to identical sequences in exon 1 of the human *COL1A1* and mouse *Coll1a1* genes (BS84). One 3'-primer was directed to sequences in exon 2 of the mouse *Coll1a1* gene (BS81), and a second 3'-primer was directed to sequences in exon 6 of the

Abbreviation used; RT, reverse transcription.

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human *COL2A1* gene, upstream of the primers used for RT (BS93). Conditions for PCR were 94 °C for 1 min, 60 °C for 2 min and 72 °C for 20 s, for a total of 16 cycles. The products were separated by electrophoresis in an 8 % (w/v) polyacrylamide gel containing 2 M urea. The gel was dried and exposed to X-ray film.

Hybridization of mRNA *in situ*

Two probes were used for *in situ* hybridization. The first one was a 766 bp fragment [5] subcloned in a plasmid (p-Bluescript KS) and derived from a human $\alpha 1(I)$ collagen cDNA (Hf-404) encoding the CNBr peptide CB7 (residues 524 to 974). The second probe was a 405 bp *HaeIII/DraI* fragment subcloned in the same plasmid and derived from a mouse cDNA coding for the 3' untranslated region of the $\alpha 1(II)$ procollagen mRNA [6]. To synthesize the ³⁵S-labelled cRNA probes, T3 or T7 RNA polymerase was used.

In situ hybridization was carried out as described by Le Guellec et al. for use of the same cDNA probes [7]. Tissue sections were cleared in two washes of xylene, rinsed twice in absolute ethanol and air dried. After washing in PBS for 5 min, sections were treated with 5 µg/ml proteinase K for 30 min at 37 °C and rinsed twice in PBS. Sections were prehybridized for 1 h at room temperature in 4 × SSC (1 × SSC = 0.15 M NaCl/15 mM sodium citrate), 1 × Denhardt's solution and 125 µg/ml salmon sperm DNA. Hybridization was performed for 16 h at 50 °C in 50 % deionized formamide, 4 × SSC, 10 % (w/v) dextran sulphate, 1 × Denhardt's solution, 125 µg/ml salmon sperm DNA and 10 mM dithiothreitol in the presence of 10⁸ c.p.m./ml radiolabelled cRNA probe. The sections were washed in 50 % formamide in 2 × SSC. Mismatched probes were digested by incubating the sections with 10 µg/ml RNase A in 1 × SSC for 30 min at 37 °C. The slides were rinsed for 30 min each at 50 °C in 1 × SSC, 0.5 × SSC, 0.25 × SSC and 0.1 × SSC. Sections were then dehydrated, dipped in Ilford-K5 Nuclear Track emulsion (Ilford, Paramus, NJ, U.S.A.) and exposed for approx. 8 days at 4 °C.

Electron microscopy

Tissue samples from control and BSG-2 transgenic mouse lines were fixed in 2 % (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, post-fixed in buffered osmium tetroxide and embedded in Spurr's epoxy resin after ethanol dehydration. Ultra-thin sections were collected on formvar-coated copper grids, contrasted in uranyl acetate and lead citrate and viewed by transmission electron microscopy (Philips CM120) at the Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie, Claude Bernard University, Lyon, France.

Immunofluorescence staining

Tissue samples from control and BSG-2 transgenic mouse lines were fixed in 4 % paraformaldehyde in PBS (pH 7.2) for 24 h and washed with PBS for 24 h before freezing. Cryosections on coated glass slides [8] were air dried for 30 min before PBS rehydration. To partially disrupt the matrix of proteoglycans, sections of xiphoid were exposed for 1 h to 0.5 % (w/v) hyaluronidase (type V; Sigma). After rinsing, the sections were blocked with 1 % (w/v) BSA in PBS. Samples were incubated with a rabbit polyclonal antibody either to human type II collagen (Institut Pasteur, Lyon, France) or to mouse type I collagen (Chemicon International Inc., Temecula, CA, U.S.A.) for 2 h, followed by FITC-conjugated goat anti-rabbit secondary

antibody (Biosys, Compiegne, France). Control sections were incubated in 1 % (w/v) BSA in PBS alone or in secondary antibody alone. Each step was performed at room temperature. The specimens were examined and photographed using a Zeiss Universal microscope equipped for epifluorescence.

Assays for type II collagen in transgenic mice

Skin samples of about 800 mg were taken from 4-week-old mice and washed twice with cold PBS containing 1 mM *p*-amino-benzamidine and 5 mM *N*-ethylmaleimide. The skin was pulverized in liquid N₂ and suspended in 10 vol. of 0.5 M acetic acid adjusted to pH 3 with HCl. The sample was digested with pepsin (1:20, w/w) at 4 °C for 24 h. The sample was centrifuged at 13000 *g* for 2 h and the collagen in the supernatant was precipitated with 1 M NaCl. The pellet was washed with ice-cold water and resuspended in 50 mM Tris/HCl (pH 7.4), 1 M NaCl, 0.02 % (w/v) NaN₃. The collagen was assayed with a colorimetric assay (Sircol Collagen Assay Kit; Biocolor Ltd., Belfast, N. Ireland). The pepsin-digested collagen was dialysed against 75 mM Tris/HCl (pH 7.8), 2 M urea, 0.02 % (w/v) NaN₃, and chromatographed on an anion exchange column (Q-Sepharose; Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. Proteins bound to the column were eluted with a buffer containing 1 M NaCl. Column fractions proteins were separated by SDS/PAGE, the gels were stained with Coomassie Blue and the bands were assayed by densitometry.

To prepare CNBr fragments, the bands identified as containing collagen α -chains were cut from the gel. The samples were cleaved with CNBr as described previously [9] and the CNBr-peptides were separated by electrophoresis on a 12.5 % polyacrylamide gel containing 0.5 M urea. Recombinant type II collagen [10] was used as a marker.

Synthesis and secretion of type I procollagen and type II procollagen by cultured skin fibroblasts from transgenic mice

Skin fibroblasts were prepared from 2-day-old BSG-2 transgenic mice by incubating minced pieces of mouse skin with Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal-bovine serum in a 100-mm² cell culture dish. Confluent primary cultures were treated with trypsin and transferred to flasks (Falcon T-175). To collect proteins secreted into the media, the cell layer was washed with PBS and serum-free media containing 40 µg/ml L-ascorbic acid phosphate (Mg salt, *n*-hydrate) (Wako, Japan) was added. After 24 h, 250 ml of media was collected from five flasks of cells. Proteins were precipitated with 176 mg/ml of (NH₄)₂SO₄ and the precipitate was collected by centrifugation at 15000 *g* for 1 h. The pellet was suspended in 0.1 M Tris/HCl (pH 7.4), 0.4 M NaCl, 25 mM EDTA, 0.02 % (w/v) NaN₃. The proteins were concentrated to a final volume of 0.5 ml by ultrafiltration (ULTRAFREE®-MC; Millipore, Bedford, MA, U.S.A.) with an exclusion size of 100 kDa. Procollagens were analysed by Western blot using rabbit polyclonal antibodies to the C-propeptide of the pro $\alpha 1(I)$ chain of type I procollagen (gift of Dr Larry Fisher, National Institute of Dental Research, National Institutes of Health, Bethesda, MD, U.S.A.) or guinea pig polyclonal antibodies to the C-telopeptide of the pro $\alpha 1(II)$ chain of human type II collagen [4,11]. The secondary antibodies were anti-rabbit or anti-guinea pig IgG, conjugated to horseradish peroxidase (Sigma). The protein bands were detected by chemiluminescence (ECL®; Amersham, Arlington Heights, IL, U.S.A.).

Northern-blot assays

Poly(A)-enriched RNA was isolated from mouse cultured skin fibroblasts using oligo-dT cellulose from a commercial kit (FastTrack 2.0 Kit; Invitrogen, San Diego, CA, U.S.A.). To prepare Northern blots, 2 µg of the poly(A)-enriched RNA were loaded per lane. After electrophoresis, the RNA was transferred on to a nylon filter (Hybond-N+; Amersham). To prepare a probe, genomic DNA from human skin fibroblasts was amplified by PCR using the primers Anti-1 (CTG TTA TCG AGT ACC GGT CAC AG) and Anti-2 (GAT CAC TCA GAT TGA TCG TGT GC). The 1017 bp PCR product began with nucleotide 26 in exon 52 of the *COL2A1* gene and extended 895 bp beyond the codon for termination of translation. In addition to the sequences indicated, the 5'-end of Anti-1 contained four random nucleotides followed by a site for the restriction enzyme *Bam*HI. The 5'-end of Anti-2 contained four random nucleotides followed by a site for the restriction endonuclease *Hind*III. The PCR products were digested with *Bam*HI and *Hind*III and cloned into a plasmid vector for RNA synthesis (pGEM-7Zf(+); Promega, Madison, WI, U.S.A.). To generate the RNA probe, the plasmid was linearized with *Sac*I and a T7 RNA polymerase was used (Riboprobe In Vitro Transcription System; Promega). To label the product, the reaction was carried out with undiluted ³²P-labelled αCTP (3,000 Ci/mmol; NEN-DuPont, Boston, MA, U.S.A.). Hybridization was performed according to manufacturer's protocol (Promega).

Transcription and translation of COL1A1–COL2A1 *in vitro*

To prepare the cDNA construct for the hybrid gene, mRNA was isolated from cultured skin fibroblasts from transgenic line BSG-2. The mRNA was reverse transcribed using random hexamers (Superscript Preamplification System; Gibco BRL). The 5'-half of the cDNA was first amplified with the primer pair M1F (5'-GAC GGG AGT TTC TCC TCG GGG TCG G) and 32-R (5'-AGA TGC ACC TTT GGG ACC ATC AGT), followed by re-amplification with the nested primer pair, Sph-F (5'-GGA GAG CAT GCA AAG AGT CTA CAT GTCTAG GGT C) and Bam-R (5'-CAC GAG GCC AGG GGA TCC AGC TTC ACC GGG AAC), to generate a 1925 bp product extending from the 5'-untranslated region of *COL1A1* to exon 30 of the cDNA for *COL2A1*. The second half of the cDNA was first amplified with the primer pair F24 (CTT CCT GGA GCC CGG GGT CTC ACT) and IH48 (5'-TCA GGT CAG CCT TTC AGT GC), followed by re-amplification with the nested primer pair, Bam-F (GTT CCC GGT GAA GCT GGA TCC CCT GGC CTC GTG) and Kpn-R (5'-GTA CTT GGT ACC TTT GGG TTT GCA ACG G), to generate a 2448 bp product extending from exon 30 to the 3'-untranslated region of the cDNA for *COL2A1*. *Sph*I, *Bam*HI or *Kpn*I restriction sites were added to the 5'-ends of the nested primers to facilitate ligation of the two halves of the cDNA.

To enhance translation efficiency of the cDNA construct, the pSPUTK vector (Stratagene) was modified by the addition of a *Sac*I–*Eco*RI fragment containing a poly(A) tail from the pSP64 vector (Promega). The construct for *in vitro* transcription/translation was assembled by restriction digestion of the PCR products followed by a three-way ligation into the modified pSPUTK vector. The cDNA construct was sequenced by automated instrumentation using vector-specific and internal primers (ABI PRISM 377 Sequencer, and ABI PRISM Dye Terminator Cycle Sequencing Ready Kit with AmpliTaq DNA polymerase, FS, Perkin Elmer). Coupled transcription/translation of the construct *in vitro* was performed using 1 µg of circular plasmid with SP6 polymerase in a 50 µl reaction mixture, according to

manufacturer's protocol (TNT Coupled Reticulocyte Lysate System; Promega). Translation products were analysed by Western blotting as described above.

RESULTS

Level of mRNA from the hybrid COL1A1–COL2A1 gene in transgenic mice

Previously, two independent lines of transgenic mice were prepared with the *COL1A1–COL2A1* hybrid construct [3]. In the present study, two additional lines were prepared. To assay mRNA expression of the transgene, a quantitative RT-PCR assay was used in which one primer was specific for exon 1 of both the human and mouse *COL1A1* genes, a second primer was

Figure 1 RT-PCR assay for expression of the *COL1A1–COL2A1* hybrid transgene

(A) Linearity of the mRNA assay. Total RNA from transgenic mouse skin was mixed at different ratios with total RNA from wild-type mouse skin. The ratio of mRNA from the *COL1A1–COL2A1* transgene to the mRNA from the mouse *Col1a1* was measured by RT-PCR, from a total of 3 µg of RNA. (B) *COL1A1–COL2A1* expression as mRNA in skin and bone of four transgenic and one wild-type mouse line. 232 bp: endogenous mouse *Col1a1*. 189 bp: *COL1A1–COL2A1*.

Figure 2 Transmission electron microscopy of collagen fibrils from tendon, skin and xiphoid

(A and B) Tendon from transgenic mouse. (C and D) Tendon from control mouse. (E) Skin from transgenic mouse. (F) Xiphoid from transgenic mouse. (G) Perichondrium from transgenic mouse. Scale bars, 300 nm.

specific for exon 2 of the mouse *Col1a1* gene and a third primer was specific for exon 6 of the human *COL2A1* gene [3]. The assay was linear over a ten-fold range when the ratio of transgenic mRNA to control (wild-type) RNA was varied from 0.1 to 1.0 (Figure 1A), and when the total amount of mRNA was varied from 1.2 to 3.8 μ g (results not shown). The four cell lines expressed various levels of the construct as mRNA (Figure 1B). The values of human *COL2A1* mRNA expressed as a percentage of mouse *Col1a1* mRNA were 3.1% in the BSG-1 line and 35–45% in the BSG-2 line. Previously, values of 5.0 and 30% were reported for these two lines respectively [3]. The values for the two new lines were 12% for BSG-3 and 30% for BSG-4. Cultured skin fibroblasts were prepared from the BSG-2 line and control mice, and poly(A)-enriched RNA was analysed by Northern blotting using an antisense cRNA probe for type II procollagen. Type II procollagen mRNA was detected at high levels in skin fibroblasts from the line BSG-2, but no signal was detected in the control skin fibroblasts (results not shown).

The mice from the BSG-1, BSG-2 and BSG-3 lines did not display any gross phenotype; they were normal in appearance, size, growth rate, behaviour and mating patterns. The mice from the line BSG-4, however, demonstrated a spontaneous circling

behaviour. The transgene mRNA expression levels in the BSG-4 line were lower than in the BSG-2 line, which did not produce the circling phenotype; therefore the circling phenotype was probably produced by an insertion-site mutation. Heterozygous mice of the BSG-4 line circled 1–2 times/s in 15 s bursts, followed by a pause of a few seconds. Two homozygous mice from the BSG-4 line, obtained from matings of heterozygous mice, circled and also exhibited severe gait disturbances. Both were about half the size of littermates and they died at 20–22 days of age.

Morphology of transgenic connective tissues

Transmission electron microscopy of several transgenic mouse tissues indicated that collagen fibrils were normal in morphology. Essentially, no discernible differences in diameter were detected between specimens from transgenic mice and controls. The large fibrils (up to 450 nm in diameter), observed in tendon from transgenic (Figures 2A and 2B) and wild-type mice (Figures 2C and 2D), were characteristic of normal type I collagen. Likewise, the small fibrils (20 nm in diameter) of type II collagen, seen in cartilage from transgenic mice (Figure 2F), were similar to normal fibrils of type II collagen in controls (results not shown).

Figure 3 Hybridization *in situ* for mRNAs for type I and type II collagens in teeth and mandible

(A) Bright-field section from transgenic mouse. (B) *In situ* hybridization with cRNA for pro α 1(I) chains on the same section as shown in (A). (C) *In situ* hybridization with cRNA for pro α 1(II) chains on the transgenic section serial to that shown in (A and B). (D) Bright-field of section from control mouse. (E) *In situ* hybridization with cRNA for pro α 1(I) chains on the same section as in (D). (F) *In situ* hybridization with cRNA for pro α 1(II) chains on control section serial to that in (D) and (E). Magnification, $\times 70$.

Also, type I collagen fibrils in skin (Figure 2E) and perichondrium (Figure 2G) from transgenic mice were the same as normal type I collagen fibrils in controls (results not shown). In addition, electron microscopy of cultured skin fibroblasts from the transgenic mice did not reveal any distention of the cisternae of the rough endoplasmic reticulum or other morphologic changes (results not shown).

Hybridization *in situ* for mRNA expression

Hybridization *in situ* with a probe that cross-reacted with both mouse and human mRNA for type II procollagen demonstrated that the hybrid *COL1A1-COL2A1* gene in the BSG-2 line was expressed as mRNA in the same cells as the endogenous *Colla1* gene. The level of the human *COL2A1* mRNA in odontoblasts of transgenic mice (Figure 3C) was roughly equal to the endogenous mouse *Colla1* mRNA (Figure 3B). Similarly, mRNA from the hybrid *COL1A1-COL2A1* gene was seen in the same regions of skin from transgenic mice as mRNA from the endogenous *Colla1* gene, with particularly high levels of mRNA

in the upper layer of the dermis (results not shown). In addition, *COL2A1* mRNA was highly expressed in fibroblasts surrounding vibrissae. Sections of the head (Figure 4) indicated that *COL2A1* mRNA from the hybrid *COL1A1-COL2A1* gene co-localized with mRNA from the endogenous mouse *Colla1* gene in the bone of the mandible (Figures 4E and 4F). Also, endogenous *Col2a1* mRNA was detected only in cartilage of control mice (Figure 4B), whereas mRNA from the hybrid *COL1A1-COL2A1* gene was detected in both cartilage and bone of transgenic mice (Figure 4C). Endogenous *Colla1* mRNA was present in bone of both transgenic (Figure 4E) and control mice (results not shown). The cDNA probe for pro α 1(II) mRNA did not cross-react with mRNA from the *Colla1* gene, since *COL1A1-COL2A1* mRNA was not detected in odontoblasts (Figure 3F) or bone (Figure 4B) of control mice.

Localization of type I and type II collagen by immunofluorescence

An attempt was made to detect type II procollagen in tissues rich in type I collagen by immunohistochemistry. There was little

Figure 4 *In situ* hybridization for mRNAs for type I and type II collagens in bone and cartilage of skull

(A) Bright-field section of bone from a control mouse. (B) *In situ* hybridization with cRNA for pro α 1(II) chains on same section as in (A). (C) *In situ* hybridization with cRNA for pro α 1(II) chains on a comparable section of bone from a transgenic mouse. (D) Bright-field section of mandible from a transgenic mouse. (E) *In situ* hybridization with cRNA for pro α 1(I) chains on same section as in (D). (F) *In situ* hybridization with cRNA for pro α 1(II) chains on section serial to that in (D) and (E). Magnification, $\times 70$.

evidence of inappropriate expression of the protein (Figure 5). There was a slight staining of dermis with antibodies to type II collagen (Figure 5B), but there was also some non-specific labelling in control samples (Figure 5C). Also, type II collagen was readily detected in xiphoid cartilage (Figure 5E) but not in the xiphoid perichondrium, which was readily stained for type I collagen (Figure 5F).

Analysis of skin for the presence of type II collagen

Because type II collagen was not detected in tissues synthesizing type I by immunohistochemistry but had been detected previously by Western-blot assays [3], an attempt was made to assay chemically the amount of type II collagen in skin. Collagens were extracted from the skin of transgenic and normal mice by pepsin digestion. Collagen α 1(II) chains co-migrate with α 1(I) chains in SDS/PAGE gels and, therefore, can be detected in samples

containing type I collagen as an increase in the expected 2:1 ratio of α 1/ α 2 chains [11]. No increase in the ratio was detected by densitometry of the gels from the transgenic mice (Figure 6). The collagens were purified by ion-exchange chromatography (Q-Sepharose; Pharmacia) under conditions in which type I collagen was recovered in the void volume and type II collagen, along with the homotrimeric form of type I collagen, were eluted with 1 M NaCl (A. Fertala, unpublished work). Densitometry of the α 1/ α 2 chain ratio in the eluted peak indicated that α 1(II) chains of type II collagen were present in the sample from the transgenic mouse. However, the amount of type II collagen was less than 2% of the amount of type I collagen recovered from the column.

To confirm the presence of a small amount of type II collagen in the eluted peak, the band of α 1 chains in the eluted fraction (lane 3 in Figures 6A and 6B) was digested with CNBr, and the peptides recovered from transgenic mice (lane 4 in Figure 7)

Figure 5 Immunofluorescent staining with antibodies to type I and type II collagens

Only transgenic tissues are shown. (A) Skin with anti-mouse type I antibody. (B) Skin with anti-human type II antibody. (C) Skin control with secondary antibody only. (D) Xiphoid control with secondary antibody only. (E) Cartilage matrix of xiphoid with anti-human type II antibody. (F) Xiphoid perichondrium with anti-mouse type I antibody. e, epidermis; d, dermis; h, hair papilla; c, cartilage; open arrow, perichondrium. Scale bar, 40 μ m.

Figure 6 Coomassie-Blue-stained gels of pepsin-treated collagens separated on a Q-Sepharose column

(A) Collagens from control mouse skin. (B) Collagens from transgenic mouse skin. Lanes 1, total protein; lanes 2, void volume; lanes 3, proteins eluted with 1M NaCl. Symbols used: γ , γ -chains, which comprise three covalently-linked α chains of collagen; β , β -chains which comprise two covalently-linked α chains of collagen; α_1 , the $\alpha_1(I)$ chain of type I collagen; α_2 , the $\alpha_2(I)$ chain of type I collagen.

Figure 7 SDS/PAGE of CNBr peptides derived from α_1 bands

Lane 1, void volume from control skin; lane 2, void volume from transgenic skin; lane 3, peak fraction from control skin eluted with 1 M NaCl; lane 4, peak fraction from transgenic skin eluted with 1 M NaCl; lane 5, recombinant type II collagen. CB 7, 8, 6 and 3 are peptides derived from $\alpha_1(I)$. CB 10, 11, 9 and 7 are peptides derived from $\alpha_1(II)$.

included characteristic fragments from $\alpha_1(II)$ chain of type II collagen not found in CNBr peptides from a control mouse (lane 3 in Figure 7).

Structure and translation *in vitro* of mRNA from cultured skin fibroblasts

To search for mutations that might prevent translation, mRNA-derived cDNA was prepared from cultured skin fibroblasts from the BSG-2 line of the transgenic mice. The cDNA was cloned and a clone containing the COL1A1–COL2A1 sequences was sequenced. The cloned cDNA was 4.4 kb and extended from 29 bp upstream of the initiation codon to 54 bp beyond the termination codon. The additional sequences between the termination codon and the polyadenylation site were analysed by sequencing an RT-PCR product prepared with primers of random hexamers. No mutations from the wild-type sequence

Figure 8 Western blot to detect polypeptide chains obtained by coupled translation and transcription of mRNA-derived cDNA from skin fibroblasts of the BSG-2 transgenic line

Protein from the coupled reticulocyte lysate system was separated by PAGE, electroblotted and the blot was reacted with a polyclonal antibody specific for the C-telopeptide of human type II collagen. Lane 1, protein extracted from tail of a BSG-2 mouse; lane 2, 10 μ l of the reaction mixture from the transcription/translation system incubated with 1 μ g of mRNA-derived cDNA from skin fibroblasts from the BSG-2 line; lane 3, negative control consisting of 10 μ l of reaction mixture incubated without added cDNA.

[12,13] were found. Also, the cDNA clone containing the COL1A1–COL2A1 sequences was transcribed and translated in a coupled reticulocyte system into full-length pro α 1(II) chains of type II procollagen (Figure 8).

DISCUSSION

Because of the distinct differences in the tissue distribution and in the structure and function of type II and type I collagen [14,15], we expected that an obvious phenotype would be produced in transgenic mice expressing the human COL2A1 gene in tissues which normally contain large amounts of type I collagen but no type II collagen. However, no gross or microscopic changes in the matrix were observed, even in the two lines of transgenic mice in which the levels of mRNA for type II procollagen were 30–45% of the level of mRNA for pro α (I) chains of type I procollagen. Previous studies with highly-sensitive Western-blot assays in the BSG-2 transgenic line demonstrated that the inappropriately expressed mRNA was translated into low levels of type II procollagen and that the type II procollagen was processed to α 1(II) and crosslinked α 1(II) chains of type II collagen [3]. The results of the present study, however, indicated a marked discrepancy between the mRNA levels and the rates of protein synthesis. No type II collagen from the exogenous gene was detected by immunofluorescence of tissues, and less than 2% of the total collagen in transgenic mouse skin was type II collagen. However, the type II collagen recovered from mice was triple-helical, since it resisted pepsin digestion. Also, it generated the correct pattern of cyanogen bromide fragments. The results, therefore, demonstrated that no phenotype was produced in the transgenic mice, because there was a selective down-regulation of inappropriate synthesis of type II procollagen in cells that had high steady-state levels of the homologous mRNAs for both type I and type II procollagen.

The results here are surprising because the same hybrid COL1A1–COL2A1 gene used to prepare the transgenic mice was shown previously [4] to cause synthesis and secretion of triple-helical type II procollagen in stably transfected mouse NIH 3T3

fibroblasts that synthesize type I collagen. The integrity of the transgene was further verified, in the present work, by demonstrating that the mRNA for type II procollagen, expressed in the transgenic mice, had the wild-type sequence. Also, the mRNA-derived cDNA from the transgenic mice was translated into pro α 1(II) chains of type II procollagen in a coupled *in vitro* transcription/translation system.

The highly selective post-transcriptional regulation found in the present study is analogous to observations in several other systems. In ferritin-sensitive cells, translation of a series of mRNAs is post-transcriptionally regulated by the binding of iron to iron-responsive elements in the 5'-ends of the RNA transcripts [16]. In tumour cells, translation of mRNA for vascular endothelial growth factor is down-regulated by overexpression of the tumour suppressor gene for von Hippel-Lindau disease [17]. During cell proliferation, the levels of mRNAs for several annexins remain constant, but translation of the mRNAs varies about 2-fold during the S- and G₂M-phases [18,19]. Except for the iron response in ferritin-sensitive cells, the mechanisms of translation regulation in these systems has not been defined. The results of the present study provide another intriguing example of post-transcriptional regulation.

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REFERENCES

- Piez, K. A. (1984) In *Extracellular Matrix Biochemistry* (Piez, K. A. and Reddi, H. A., eds.), pp. 1–40, Elsevier Science Publishing, New York.
- Prockop, D. J. and Kivirikko, K. I. (1995) *Annu. Rev. Biochem.* **64**, 403–434.
- Sokolov, B. P., Ala-Kokko, L., Dhulipala, R., Arita, M., Khillan, J. S. and Prockop, D. J. (1995) *J. Biol. Chem.* **270**, 9622–9629.
- Ala-Kokko, L., Hyland, J., Smith, C., Kivirikko, K. I., Jimenez, S. A. and Prockop, D. J. (1991) *J. Biol. Chem.* **266**, 14175–14178.
- Bernard, M. P., Chu, M.-L., Myers, J. C., Ramirez, F., Eikenberry, E. F. and Prockop, D. J. (1995) *Biochemistry* **22**, 5213–5223.
- Metsäranta, M., Toman, D., de Crombrughe, B. and Vuorio, E. (1991) *Biochim. Biophys. Acta* **1089**, 241–243.
- Le Guellec, D., Mallein-Gerin, F., Treilleux, I., Bonaventure, J., Peysson, P. and Herbage, D. (1994) *Histochem. J.* **2**, 695–704.
- Rentrop, M., Knapp, B., Winter, H. and Schweizer, J. (1986) *Histochem. J.* **18**, 271–276.
- Sokolov, B. P., Sher, B. M. and Kalinin, V. N. (1989) *Anal. Biochem.* **176**, 365–367.
- Fertala, A., Sieron, A. L., Ganguly, A., Li, S.-W., Ala-Kokko, L., Anumula, K. R. and Prockop, D. J. (1994) *Biochem. J.* **298**, 31–37.
- Jimenez, S. A., Ala-Kokko, L., Prockop, D. J., Merryman, D. F., Shepard, N. and Dodge, G. R. (1997) *Matrix Biol.* **16**, 29–39.
- Tromp, G., Kuivaniemi, H., Stacey, A., Shikata, H., Baldwin, C. T., Jaenisch, R. and Prockop, D. J. (1988) *Biochem. J.* **253**, 919–922.
- Baldwin, C. T., Reginato, A. M., Smith, C., Jimenez, S. A. and Prockop, D. J. (1989) *Biochem. J.* **262**, 521–528.
- Kuivaniemi, H., Tromp, G. and Prockop, D. J. (1997) *Hum. Mut.* **9**, 300–315.
- Byers, P. H. (1983) In *Connective Tissue and Its Heritable Disorders* (Royce, P. M. and Steinmann, B., eds.), pp. 317–350, Wiley-Liss, New York.
- Kim, H. Y., LaVaute, T., Iwai, K., Klausner, R. D. and Rouault, T. A. (1996) *J. Biol. Chem.* **271**, 24226–24230.
- Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J. R., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D. and Linehan, W. M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10589–10594.
- Schlaepfer, D. D. and Haigler, H. T. (1990) *J. Cell. Biol.* **111**, 229–238.
- Raynal, P., Pollard, H. B. and Srivastavi, M. (1997) *Biochem. J.* **322**, 365–371.