Journal of Cellular Biochemistry 66:210–218 (1997)

Localization of Silencer and Enhancer Elements in the Human Type X Collagen Gene

Frank Beier,[1,2] Silvia Vornehm,[1] Ernst Pöschl,[1] Klaus von der Mark,[1] and Mikko J. Lammi[3]*

[1]Institut für Experimentelle Medizin, Universität Erlangen-Nürnberg, Erlangen, Germany
[2]Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada
[3]Department of Anatomy, University of Kuopio, Kuopio, Finland

Abstract Collagen type X is a short, network-forming collagen expressed temporally and spatially tightly controlled in hypertrophic chondrocytes during endochondral ossification. Studies on chicken chondrocytes indicate that the regulation of type X collagen gene expression is regulated at the transcriptional level. In this study, we have analyzed the regulatory elements of the human type X collagen (Col10a1) by reporter gene constructs and transient transfections in chondrogenic and nonchondrogenic cells. Four different promoter fragments covering up to 2,864 bp of 5′-flanking sequences, either including or lacking the first intron, were linked to luciferase reporter gene and transfected into 3T3 fibroblasts, HT1080 fibrosarcoma cells, prehypertrophic chondrocytes from the resting zone, hypertrophic chondrocytes, and chondrogenic cell lines. The results indicated the presence of three regulatory elements in the human Col10a1 gene besides the proximal promoter. First, a negative regulatory element located between 2.4 and 2.8 kb upstream of the transcription initiation site was active in all nonchondrogenic cells and in prehypertrophic chondrocytes. Second, a positive, but also non-tissue-specific positive regulatory element was present in the first intron. Third, a cell-type-specific enhancer element active only in hypertrophic chondrocytes was located between −2.4 and −0.9 kb confirming a previous report by Thomas et al. [(1995): Gene 160:291–296]. The enhancing effect, however, was observed only when calcium phosphate was either used for transfection or included in the culture medium after lipofection. These findings demonstrate that the rigid control of human Col10a1 gene expression is achieved by both positive and negative regulatory elements in the gene and provide the basis for the identification of factors binding to those elements. J. Cell. Biochem. 66:210–218, 1997.

Key words: collagen type X; gene regulation; calcium phosphate; reporter gene; transfection; hypertrophic chondrocytes

Type X collagen is a short-chain, homotrimeric collagen which assembles into a hexagonal meshwork [Schmid and Linsenmayer, 1985a; Kwan et al., 1991]. It is transiently expressed in a spatially and developmentally tightly controlled manner in the hypertrophic zone of epiphyseal growth plates in long bones, ribs, and vertebrae during endochondral ossification [Gibson and Flint, 1985; Schmid and Linsenmayer, 1985a; Ninomiya et al., 1986; Reichenberger et al., 1991]. Under pathological conditions, type X collagen is found in osteoarthritic cartilage [Hoyland et al., 1991; von der Mark et al., 1992, 1995; Aigner et al., 1993; Walker et al., 1995] and in chondrosarcomas [Aigner et al., in press]. Although the function of type X collagen is still under debate at present, its close association with the sites of initial cartilage calcification and metaphyseal bone marrow invasion [Gibson and Flint, 1985; Schmid and Linsenmayer, 1985b] suggests a major role in the endochondral ossification. Previous studies on the regulation of the type X collagen gene in the chicken indicated that this gene is regulated at the level of transcription [Lu Valle et al., 1989]. In human, mouse, and chicken, the gene is known to be composed of only three exons, with a large exon 3 coding for more than 90% of the protein [Ninomiya et al., 1986; Lu Valle et al., 1988; Thomas et al., 1991a,b; Reichenberger et al., 1992; Apte and

Contract grant sponsor: Deutsche Forschungsgemeinschaft, contract grant number Ma 534/11-1; contract grant sponsors: Academy of Finland, Saastamoinen Foundation, University of Kuopio, and Farmos Research and Science Foundation.

*Correspondence to: Mikko Lammi, Department of Anatomy, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland. E-mail: mikko.lammi@uku.fi

Received 16 December 1996; accepted 26 March 1997

Olsen, 1993; Elima et al., 1993; Kong et al., 1993]. This genomic organization is distinct from most other collagen genes; only the α_1 and α_2 genes of the structurally related type VIII collagen show a similar condensed gene structure [Muragaki et al., 1991; Yamaguchi et al., 1991].

Negative regulatory elements in the 5′-flanking regions of the chicken Col10a1 gene were described inhibiting its expression in fibroblasts and immature chondrocytes [Lu Valle et al., 1993]. Recently, in vivo footprinting in collagen type X–expressing chick hypertrophic chondrocytes showed that a number of sites in the proximal promoter region are protected, including a fully protected TATA box, a sequence overlapping an AP-2 site, and a 9 bp region containing the sequence CACACA [Long and Linsenmayer, 1995]. Nucleotide sequence comparison of regulatory regions in the 5′ end of the chicken and mammalian Col10a1 genes, however, revealed significant differences in the promoter sequences among the species. Homology is obvious only in the 120 nucleotides proximal to the transcription initiation site [Beier et al., 1996], indicating that avian and mammalian Col10a1 genes may be regulated by different mechanisms.

Recently, an enhancer element in the region 2,400 to 900 bp upstream of the transcription initiation site of human Col10a1 gene was reported which was effective only in hypertrophic chondrocytes [Thomas et al., 1995]. Here we generated various reporter gene constructs extending up to −2,864 bp from the transcription initiation site, including or excluding the first intron, in order to identify further positive and negative regulatory elements in this gene. By transfection of hypertrophic and prehypertrophic chondrocytes and of nonchondrogenic cells we provide evidence for the presence of 1) a strong silencer element between 2,410 and 2,864 bp upstream of the transcription start site which repress the transcription of the type X collagen in cells other than hypertrophic chondrocytes, 2) a general, unspecific enhancer in the first intron, and 3) a positive regulatory element enhancing Col10a1 expression specifically in hypertrophic chondrocytes; this was effective only when hypertrophic chondrocytes were transfected in the presence of calcium phosphate. Our findings indicate a complex control of the human Col10a1 gene transcription by both positive and negative regulatory elements and thus may contribute to our understanding of the mechanism of type X collagen regulation in cartilage development and degeneration.

MATERIALS AND METHODS

Materials

The luciferase reporter gene vectors used for transfection experiments (pGL2 basic, pGL2 control, and pGL2 promoter), a β-galactosidase reporter gene plasmid (pSVβ-Gal), and luciferase reporter gene assay system were provided by Promega (Madison, WI). *Vent*™ DNA polymerase was purchased from New England Biolabs GmbH (Schwalbach, Germany). SureClone Ligation, Ready-To-Go DNA Labelling, and T^7 Sequencing kits were obtained from Pharmacia (Uppsala, Sweden), a β-galactosidase ELISA kit and testes hyaluronidase from Boehringer-Mannheim (Mannheim, Germany), and Lipofectin™, DMEM, and Opti-MEM media from GibcoBRL (Paisley, UK). *AmpliTaq* DNA polymerase was delivered by Perkin Elmer Applied Biosystems GmbH (Weiterstadt, Germany). Plasmids used for the transfections were purified with a Qiagen Plasmid Kit (Qiagen, Chatsworth, CA). The following cell lines were used: HT1080 fibrosarcoma and 3T3 fibroblasts obtained from American Type Culture Collection (Rockville, MD), while a WT5-5 chondrocytic cell line derived from a *c-fos* overexpressing transgenic mouse [Wang et al., 1993] and MCTs mouse endochondral chondrocytes immortalized by a temperature-sensitive simian virus 40 large tumor antigen [Lefebvre et al., 1994] were kindly donated by Dr. Z.-Q. Wang and Dr. E. Wagner and by Dr. V. Lefebvre and Dr. B. de Crombrugghe, respectively.

Construction of the Reporter Gene Plasmids

The luciferase reporter gene plasmids used in transfections were constructed by inserting fragments of the human Col10a1 promoter region together with 31 nucleotides of the first exon into the vector pGL2 basic to yield the plasmids pGLXH3000 (−2,864 +31), pGLH2500 (−2,410 +31), pGLBH900 (−873 +31) and pGLSH500 (−460 +31), respectively (for details see Fig. 1). The numbering of the base pairs refers to the sequence as documented in GenBank accession number X98568. PCR amplification of the first intron (+31 +639) by *Vent* DNA polymerase was used to generate a novel *Hind III* restriction cleavage site within exon 2 at position +622. The PCR amplification was

started with heating the sample at 93°C for 10 min; then 32 cycles with 44°C for 1 min and elongation with 72°C for 1 min were performed. The sense primer used in the PCR was 5′-GAG GAA GCT TCA GAA AGC TG-3′, and the antisense primer was 5′-GGG TAT TTG TGG AAG CTT ATT CTC AGA TGG-3′. The PCR product was cloned into pUC18, and the resulting 591 bp *Hind III* fragment was ligated into the *Hind III* site of the plasmids pGL2 promoter, pGLBH900, and pGLXH3000 to yield the plasmids pGL-PromInt1 (+240 +831), pGLBH900Int1 (+952 +1,542), and pGLXH3000Int1 (+2,942 +3,532), respectively (Fig. 1). The correct orientation of the insert was ascertained using T^7 sequencing Kit (Pharmacia).

Cell Culture and Transfections

Primary chondrocytes were prepared from fetal calves. The tibiofemoral, humero-radioulnar, and intertarsal joints of the limbs were used to separately isolate the resting and hypertrophic chondrocytes. The cells were released by trypsin treatment (0.1% trypsin in PBS) for 30 min followed by overnight incubation with 0.05% collagenase in DMEM supplemented with 10% fetal calf serum (FCS), washed with DMEM, and seeded into 35 mm culture dishes (500,000 cells/well). Primary fibroblasts were isolated from the skin of bovine embryos. Cell lines and primary cells were kept in DMEM with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin and cultured using standard cell culture techniques.

For transfections, cell lines and primary cells were cultured in DMEM including 10% FCS, 1 mM D-pyruvate, 1 mM D-cysteine, 50 µg/ml L-ascorbate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Transfections were done in 35 mm wells with 7 µg of the reporter gene constructs using Lipofectin™ (GibcoBRL) in Opti-MEM medium according to the manufacturer's instructions. In transfections of primary chondrocytes, hyaluronidase (4 U/ml) was included in the medium in all steps after the initial seeding onto the plates to obtain better transfection efficiency [Lu Valle et al., 1993]. To normalize luciferase results for relative transfection efficiency, the cells were cotransfected with 4 µg of the plasmid pSVβ-Gal (Promega), containing the gene for β-galactosidase. The cultures were transfected for 6 h at 32°C for MCTs cells and 37°C for all the other cells in 5% CO_2; then Opti-MEM was replaced by DMEM as described above. Eighteen hours after the transfection the cells were lysed, and the cytosolic extract was tested for luciferase activity according to the manufacturer's protocol (Promega). β-galactosidase activity was determined using an *o*-nitrophenyl galactosopyranoside assay [Rosenthal, 1987]. Transfection efficiency was calculated based on the β-galactosidase activity in standard amount of cytosolic extracts, and the relative transcriptional activity was then calculated as the ratio of luciferase activity vs. the normalized β-galactosidase activity. Experiments consisting of two to three parallel cultures were repeated two to four times with chondrocytes isolated from different fetal calves, and the results are presented as mean ± SD. When the transfection efficiency was low in the hypertrophic chondrocytes, a more sensitive ELISA assay kit (Boehringer-Mannheim) was used for determination of the amount of β-galactosidase.

For the analysis of the effects of calcium and phosphate, transfections were also done by calcium phosphate coprecipitation [Sambrook et al., 1989] for 6 h at 37°C in 5% CO_2. The transfection medium was replaced by DMEM, and cells were lysed after 18 h. In some experiments, the extracellular Ca^{2+} concentration was increased by adding 5 mM $CaCl_2$ to the culture medium after removal of Lipofectin™, or calcium phosphate (without DNA) was applied to the cells after Lipofectin™ transfection in exactly the same way as used in calcium phosphate transfections.

RESULTS

For the analysis of positive and negative regulatory elements in the human Col10a1 gene, various reporter gene constructs were prepared by fusion of the luciferase gene to the 5′ end of the first exon and different upstream regions of the promoter, including or lacking the first intron (Fig. 1).

Basal Promoter Activities in Nonchondrogenic Cells, Prehypertrophic Chondrocytes, and MCTs Cells

The transcriptional regulation of human collagen type X gene was first studied in a number of different cell types not expressing collagen type X and in a mouse cell line (MCTs) reported to synthesize type X collagen [Lefebvre et al., 1994]. Primary bovine chondrocytes from the

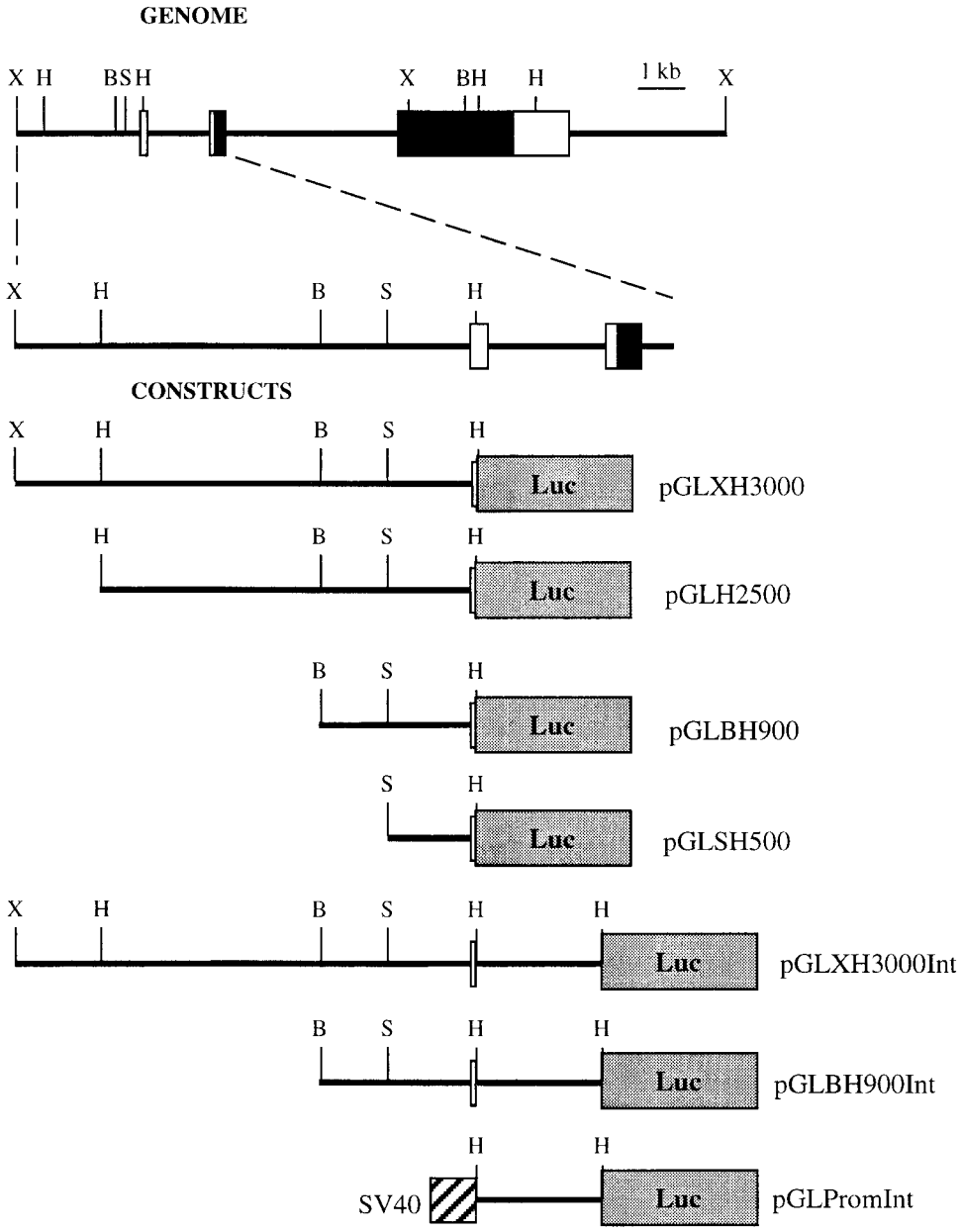

Fig. 1. Type X collagen gene reporter plasmids. Different fragments of the human type X collagen gene were cloned upstream of the luciferase reporter gene in the vector pGL2 basic (Promega). **Top:** The genomic structure of the human type X collagen gene is shown. Boxes represent exons, with the coding sequence drawn in black. Letters indicate the restriction sites used for cloning of the fragments (B, *BamHI*; S, *Ssp I*; X, *Xho I*). **Bottom:** The 5′-part of the gene is shown enlarged, and the reporter gene constructs are indicated in relation to the gene (Luc, luciferase reporter gene). For cloning of intron 1, a *Hind III* site was introduced in the 5′ end of exon 2 (see Materials and Methods for details). The SV40 promoter in pGLPromInt is derived from the pGL2 promoter (Promega).

resting zone, the murine nonhypertrophic chondrocyte cell line WT5-5, the fibroblast cell line 3T3, the human fibrosarcoma cell line HT1080, and MCTs chondrocytes were transfected with the Col10a1 promoter constructs by lipofection. As shown in Table I, in all cells types tested the shortest upstream promoter fragment of the human collagen type X gene produced a low level of reporter gene activity comparable to that of thymidine kinase promoter (pTKLuc).

Strong Silencer Element Is Active in Cells Not Expressing Type X Collagen

The clones pGLBH900 and pGLH2500 generally revealed the highest reporter gene activities, while in all cells except MCTs cells

TABLE 1. Activity of Different Type X Collagen Promoter Fragments in Various Cell Types*

Cell type	pTKLuc	pGLSH500	pGLBH900	pGLH2500	pGLXH3000
Bovine epiphyseal chondrocytes	1.00 ± 0.03	1.12 ± 0.04	1.47 ± 0.07	1.38 ± 0.05	0.43 ± 0.06
Bovine fibroblasts	1.00 ± 0.03	1.10 ± 0.06	1.96 ± 0.04	1.85 ± 0.07	0.67 ± 0.03
3T3 fibroblasts	1.00 ± 0.06	1.03 ± 0.05	1.94 ± 0.07	2.08 ± 0.08	0.68 ± 0.06
WT5-5 cells	1.00 ± 0.06	1.01 ± 0.05	2.28 ± 0.11	2.37 ± 0.09	0.77 ± 0.05
HT1080 cells	1.00 ± 0.06	1.01 ± 0.06	2.64 ± 0.05	3.07 ± 0.06	0.96 ± 0.05
MCTs cells	1.00 ± 0.03	9.02 ± 0.21	8.56 ± 0.15	6.02 ± 0.12	5.69 ± 0.06

*Several chondrogenic and nonchondrogenic cell types were transfected with pTKLuc and plasmids pGLSH500, pGLBH900, pGLH2500, and pGLXH3000. Forty hours after transfection, cells were lysed, cytosolic luciferase activity was determined, and the results were normalized to β-galactosidase activity. Transfections were performed with three parallel cultures, and experiments were repeated two to three times. Values are presented as average ± SD.

pGLXH3000 conferred about 30% of the activity of pGLBH900 and pGLH2500, suggesting the presence of a negative regulatory element. In contrast, the collagen type X–expressing MCTs cells not only revealed approximately fivefold higher reporter gene activities compared to the thymidine kinase promoter (Table I) and the simian virus 40 promoter/enhancer (pGL2 control; data not shown), but also the activity of pGLXH3000 was nearly identical to that of pGLH2500 (Table I). Taken together, these results suggested the presence of a negative element between 2,864 and 2,410 nucleotides upstream of the transcription initiation site of the human Col10a1 gene. This element was functional in all cells not expressing collagen type X but not in MCTs cells, suggesting that it may have a role in the cell-specific regulation of Col10a1 gene expression.

Cell-Specific Enhancer Is Active Only in Type X Collagen–Expressing Chondrocytes

In contrast to the results by Thomas et al. [1995] showing a strong enhancer in a reporter gene construct corresponding to our plasmid pGLH2500 after transfection of bovine primary hypertrophic chondrocytes, we did not observe such an effect with pGLH2500 in MCTs cells and hypertrophic chondrocytes when Lipofectin™ was used for transfection (Table I; Fig. 2A).

To analyze whether this discrepancy was a result of the different transfection methods, we compared the promoter activities in hypertrophic chondrocytes transfected with Lipofectin™ or calcium phosphate–precipitated DNA. In Lipofectin™-transfected bovine hypertrophic chondrocytes (with no Ca^{2+} addition), no enhancer effect was observed when pGLBH900 and pGLH2500 were compared (Fig. 2A). In contrast, when transfections were performed using the calcium phosphate coprecipitation method, pGLH2500 conferred four- to fivefold higher relative luciferase activities than pGLBH900 (Fig. 2B). In HT1080 cells, no difference between the two transfection methods was seen (data not shown).

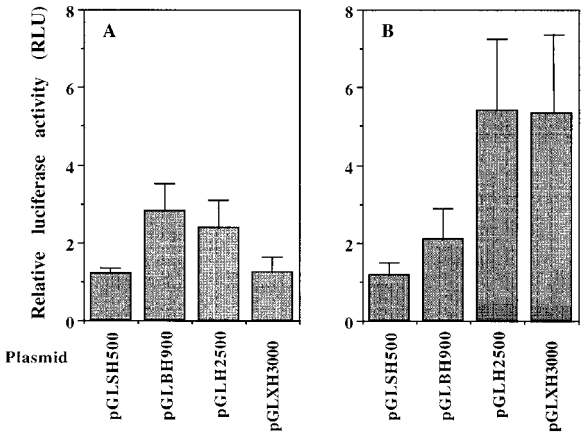

Fig. 2. A strong enhancer element is located in the human type X collagen gene promoter. Bovine hypertrophic chondrocytes were transfected with pGLSH500, pGLBH900, pGLH2500, and pGLXH3000 using Lipofectin™ (**A**) and calcium phosphate–precipitated DNA (**B**). Eighteen hours after transfection, the cells were harvested, and cytosolic luciferase activity was determined and normalized to β-galactosidase activities. A strong enhancer element is located in the region of 2,410 to 873 bp upstream of the transcription initiation site. It is active only in calcium phosphate–transfected cells. Transfections were performed with two to three parallel cultures, and experiments were repeated four times. The bars show the mean ± SD.

The presence of hyaluronidase during transfection of the hypertrophic chondrocytes usually enhanced the relative luciferase activities of plasmids pGLH2500 and pGLXH3000. Without enzyme addition, transfection efficiency was normally rather low; therefore, hyaluronidase was subsequently included in all steps of the

transfection experiments with primary chondrocytes.

Transfection With Calcium Phosphate Is Required to Reveal the 5′-Upstream Enhancer

Our data indicate that the enhancer element between positions 2,410 and 873 bp upstream the transcription initiation site is calcium phosphate–dependent (Figs. 2B, 3B), suggesting the presence of a calcium phosphate response element in the promoter. Therefore we performed additional experiments in hypertrophic chondrocytes using Lipofectin™ transfections with an additional 5 mM Ca^{2+} included in the medium after transfections. Transfection assays by the calcium phosphate coprecipitation method were performed in parallel using the same population of hypertrophic chondrocytes. Addition of Ca^{2+} in the presence of Lipofectin™ was not possible due to the precipitation of liposomes by calcium salts. When $CaCl_2$ was added after Lipofectin™ transfections at concentrations corresponding to those achieved during calcium phosphate precipitation (6.7 mM), the construct pGLH2500 showed the same relative luciferase activity (Fig. 3C) as observed in the absence of $CaCl_2$ (Fig. 3A). However, when calcium phosphate precipitate (without DNA) was added to the cultures after Lipofectin™ transfection, the relative reporter gene activity of the plasmid pGLH2500 was increased to 200–300% of the activity of the plasmid pGLBH900 (Fig. 3D), suggesting that enhancer effect of the fragment from −2,410 to −873 bp of the transcription initiation site is dependent on the calcium phosphate but not on the Ca^{2+} ion.

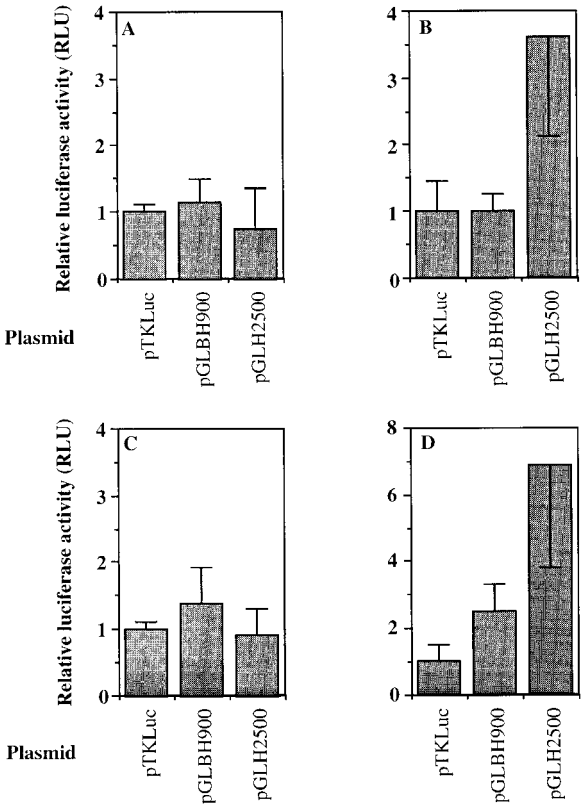

Fig. 3. Calcium phosphate transfection is required to reveal the enhancer effect. Bovine hypertrophic chondrocytes were transfected with pTKLuc, pGLBH900, and pGLH2500 using Lipofectin™ (**A**), calcium phosphate–precipitated DNA (**B**), or Lipofectin™ with subsequent incubation with $CaCl_2$ (**C**) or calcium phosphate (**D**). Eighteen hours after transfection, the cells were harvested, and cytosolic luciferase activity was determined and normalized to β-galactosidase activities. The enhancer effect of the fragment between −2,410 bp and −873 bp from the transcription initiation site was observed only when calcium phosphate was included in the cultures of hypertrophic chondrocytes. Transfections were performed with three parallel cultures, and experiments were repeated two times. The bars show the mean ± SD.

First Intron Contains an Enhancing Element

The role of the first intron in the regulation of Col10a1 gene expression was investigated with reporter gene constructs that included the first intron in the constructs pGLBH900 and pGLXH3000. Intron 1 was introduced into the *Hind III* site at the 3′ end of the human Col10a1 promoter fragment to reconfigure the genomic situation. Additionally, the first intron was cloned into a reporter gene plasmid containing the unrelated promoter of SV40 (pGL2 promoter).

Transfections with intron 1 constructs were performed in primary hypertrophic chondrocytes and HT1080 and MCTs cell lines. In all three cell types, the plasmids which included the first intron showed an increase in the relative luciferase activity when compared with respective intronless constructs, both when calcium coprecipitation (Fig. 4) and lipofection (results not shown) were used for transfections.

DISCUSSION

In this study, we describe the identification and analyses of three major *cis*-acting DNA regulatory elements in the human type X collagen gene Col10a1. We confirmed the existence of a strong, tissue-specific enhancer element between −2,410 and −873 bp from the transcription initiation site which has been reported previously [Thomas et al., 1995]. In the

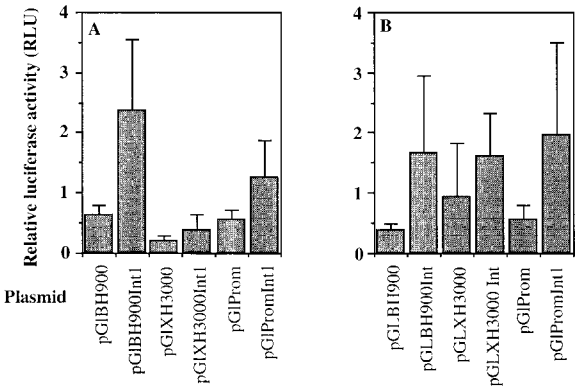

Fig. 4. A general enhancer is located in the first intron of the human type X collagen gene. HT1080 cells (**A**) and bovine hypertrophic chondrocytes (**B**) were transfected with pGL2 promoter (Promega), pGLBH900, pGLXH3000, and the same plasmids containing intron 1 of the type X collagen gene, pGLPromInt, pGLBH900Int, and pGLXH3000Int. Eighteen hours after transfection, the cells were harvested, and cytosolic luciferase activity was determined and normalized to β-galactosidase activities. Transfections were performed with three parallel cultures, and experiments were repeated three times. The bars show the mean ± SD.

first intron, another potential enhancer element was observed which, however, was not cell-specific. In the region ranging from −2,864 to −2,410 bp upstream of the transcription initiation site of the human type X collagen gene, a strong silencer element was identified which is responsible for suppression of type X collagen expression in nonhypertrophic chondrocytes and in other nonchondrogenic cells.

In hypertrophic chondrocytes, both the enhancer and silencer elements of 5′ promoter region were recognized only when calcium phosphate was used for transfection of reporter constructs. Only in the presence of calcium phosphate did the enhancer element of human collagen type X promoter (−2,410 to −873 bp) increase the transcriptional activity in hypertrophic chondrocytes by 200–300% in comparison to the basal promoter.

Some earlier reports have shown the importance of extracellular Ca^{2+} and calcium glycerophosphate for chicken collagen type X gene expression in cell culture [Thomas et al., 1990; Bonen and Schmid, 1991]. When chicken embryos were cultured in vitro under Ca^{2+}-deficient conditions, the differentiation of chondrocytes and expression of type X collagen were severely inhibited [Reginato et al., 1993]. The presence of 10 mM $CaCl_2$ greatly stimulated collagen type X expression in chicken chondrocytes [Bonen and Schmid, 1991]. However, in our studies the addition of $CaCl_2$ alone was not able to induce an enhancer effect in bovine chondrocytes. This discrepancy may either be due to species differences or may be caused by the conditions used, exposing the chondrocytes to $CaCl_2$ less than 24 h, which was the standard time used in our transfection experiments. The mechanism by which calcium phosphate stimulates transcription of the pGLH2500 and pGLXH3000 reporter gene constructs is unknown at present. One possibility is that calcium phosphate crystals may enter the cells and transmit their biological action by intracellular effects. Indeed, a rapid mineralization in cell cultures of the osteoblastic cell line UMR 106-01 BSP has been reported to occur in the presence of organophosphates or calcium, and intracellular apatite crystals were found in electron microscopic analyses [Stanford et al., 1995].

The stimulating effect of the enhancer activity of the element between −2,410 and −873 bp was much lower in our hands when compared to the report by Thomas et al. [1995]. One possible explanation for the different reporter gene activities may be variations in the ratio of proliferative chondrocytes not expressing type X collagen vs. hypertrophic chondrocytes in both studies. The differences may also be explained by the different reporter genes used, both for collagen type X promoter constructs (chloramphenicol acetyltransferase vs. luciferase), and for internal normalization of the transfection efficiency (growth hormone vs. β-galactosidase). Inconsistent results have been observed, for instance, in the studies localizing the *cis*-acting DNA elements in the promoter and first intron of the mouse procollagen α_1(I) gene using different reporter genes [Liska et al., 1994; Rossert et al., 1995; Sokolov et al., 1995]. Yet it is important to notice here that, despite the different reporter gene systems used in the study by Thomas et al. [1995] and our study, the same enhancer region was identified when similar transfection methods were applied. However, as the luciferase reporter gene is considerably more sensitive than the chloramphenicol acetyltransferase gene, we observed a significant basal transcriptional activity of the plasmid pGLBH900 in all cell types tested, while Thomas et al. [1995] did not describe such an activity. It is possible that the absolute activity of the −2,500 promoter containing the enhancer is very similar in both systems, whereas the differences in the ratios between the −873 and

the −2,410 promoters are mainly due to different basal levels obtained with shorter fragments (−460 and −873).

Based on our transfection experiments in cells not expressing Col10a1, we have identified a novel silencer element in the region ranging from −2,864 to −2,410 base pairs proximal to the transcription initiation site of the human α_1(X) collagen gene. The silencer was functional in nonchondrogeneic cells and prehypertrophic chondrocytes. In hypertrophic chondrocytes transfected with calcium phosphate coprecipitation, the silencer was not active at all. Part of the silencer effect observed after lipofection may be due to the presence of immature, proliferative chondrocytes in the chondrocyte preparation not expressing the type X collagen gene.

The first intron of human collagen type X gene acts as a weak, cell type–independent general enhancer. Intron 1 is well conserved in human and mouse type X collagen genes [Beier et al., 1996], suggesting that it may contain elements that are important for the regulation of the human type X collagen gene. Similarly, in some other collagen genes the first introns have been shown to reveal enhancer function [Rossouw et al., 1987; Bornstein et al., 1987; Horton et al., 1987]. For example a 182 bp intron 1 fragment of the mouse Col2a1 gene directs the expression of the β-galactosidase reporter gene in transgenic mice specifically to chondrocytes, with the same temporal pattern as that of the endogenous Col2a1 gene [Zhou et al., 1995]. Our data indicate, however, that the enhancer in the first intron of the human Col10a1 gene does not target the expression specifically to hypertrophic chondrocytes, since its enhancing effect was shown to be independent of the cell type investigated.

In summary, our findings indicate that the tissue-specific expression of human type X collagen in hypertrophic cartilage is controlled by both positive and negative regulatory elements present in the promoter. In the chicken, type X collagen expression seems to be controlled mainly by negative regulatory elements [Lu Valle et al., 1993; Long and Linsenmayer, 1995]. The morphology of avian long bone development, the spatial and temporal aspects of cartilage differentiation, hypertrophy, and remodelling to bone are, however, significantly different from mammalian bone development. In the developing avian bone, type X collagen–expressing hypertrophic chondrocytes contribute to a major portion of the cartilage [Schmid and Linsenmayer, 1985b], while they are restricted to only a narrow zone of endochondral ossification in the mammalian epiphysis [Reichenberger et al., 1991]. This may require different control mechanisms of type X collagen gene expression. So far the transcriptional activities of all these regulatory sites in type X collagen genes have been shown only in vitro and must be confirmed in vivo by transgenic approaches.

ACKNOWLEDGMENTS

This research was supported by grants from the Deutsche Forschungsgemeinschaft (K.vdM.; Ma 534/11-1), the Academy of Finland (M.J.L.), the Saastamoinen Foundation (M.J.L.), University of Kuopio (M.J.L.), and the Farmos Research and Science Foundation (M.J.L.).

REFERENCES

Aigner T, Reichenberger E, Bertling W, Kirsch T, Stöss H, von der Mark K (1993): Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch B Cell Pathol 63:205–211.

Aigner T, Frischholz S, Dertinger S, Beier F, Girkontaite I, von der Mark K (in press): Type X collagen expression and hypertrophic differentiation in chondrogenic neoplasias. Histochem Cell Biol.

Apte SS, Olsen BR (1993): Characterization of the mouse type X collagen gene. Matrix 13:165–179.

Beier F, Iirola E, Vuorio E, Lu Valle P, Reichenberger E, Bertling W, von der Mark K, Lammi MJ (1996): Variability in the upstream promoter and intron sequences among the human, murine and chick type X collagen genes. Matrix Biol 15:415–422.

Bonen DK, Schmid TM (1991): Elevated extracellular calcium concentrations induce type X collagen synthesis in chondrocyte cultures. J Cell Biol 115:1171–1178.

Bornstein P, McKay J, Morishima JK, Devarayalu S, Gelinas RE (1987): Regulation elements in the first intron contribute to transcriptional control of the human α_1(I) collagen gene. Proc Natl Acad Sci U S A 84:8869–8873.

Elima K, Eerola I, Rosati R, Metsäranta M, Garofalo S, Perälä M, de Crombrugghe B, Vuorio E (1993): The mouse collagen X gene: Complete nucleotide sequence, exon structure and expression pattern. Biochem J 289: 247–253.

Gibson GJ, Flint MH (1985): Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral development. J Cell Biol 101:277–284.

Horton W, Miyashita T, Kohno K, Hassell JR, Yamada Y (1987): Identification of a phenotype-specific enhancer in the first intron of the rat collagen II gene. Proc Natl Acad Sci U S A 84:8864–8868.

Hoyland JA, Thomas J, Donn R, Marriott A, Ayad S, Boot-Handford R, Grant ME, Freemont AJ (1991): Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. Bone Miner 15:151–164.

Kong RYC, Kwan KM, Lau ET, Thomas JT, Boot-Handford RP, Grant ME, Cheah KSE (1993): Intron-exon structure, alternative use of promoter and expression of the mouse collagen X gene, Col10a-1. Eur J Biochem 213:99–111.

Kwan APL, Cummings CE, Chapman JA, Grant ME (1991): Macromolecular organization of chicken type X collagen in vitro. J Cell Biol 114:597–604.

Lefebvre V, Garofalo S, Zhou G, Metsäranta M, Vuorio E, de Crombrugghe B (1994): Characterization of primary cultures of chondrocytes from type II collagen/β-galactosidase transgenic mice. Matrix Biol 14:329–335.

Liska DJ, Reed MJ, Sage EH, Bornstein P (1994): Cell-specific expression of α1(I) collagen hGH minigenes in transgenic mice. J Cell Biol 125:695–704.

Long F, Linsenmayer TF (1995): Tissue-specific regulation of the type X collagen gene. Analyses by in vivo footprinting and transfection with a proximal region. J Biol Chem 270:31310–31314.

Lu Valle P, Ninomiya Y, Rosenblum ND, Olsen BR (1988): Type X collagen gene. Intron sequences split the 5′-untranslated region and separate the coding regions for the non-collagenous amino-terminal and triple-helical domains. J Biol Chem 263:18378–18385.

Lu Valle P, Hayashi M, Olsen BR (1989): Transcriptional regulation of type X collagen during chondrocyte maturation. Dev Biol 133:613–616.

Lu Valle P, Iwamoto M, Fanning P, Pacifici M, Olsen BR (1993): Multiple negative elements in a gene that codes for an extracellular matrix protein, collagen X, restrict expression to hypertrophic chondrocytes. J Cell Biol 121: 1173–1179.

Muragaki Y, Jacenko O, Apte S, Mattei M-G, Ninomiya Y, Olsen BR (1991): The α_2(VIII) collagen gene. A member of the short chain collagen family located on chromosome 1. J Biol Chem 266:7721–7727.

Ninomiya Y, Gordon M, van der Rest M, Schmid T, Linsenmayer T, Olsen BR (1986): The developmentally regulated type X collagen gene contains a long open reading frame without introns. J Biol Chem 261:5041–5050.

Reginato A, Tuan RS, Ono T, Jimenez SA, Jacenko O (1993): Effects of calcium deficiency on chondrocyte hypertrophy and type X collagen expression in chick embryonic sternum. Dev Dyn 198:284–295.

Reichenberger E, Aigner T, von der Mark K, Stöss H, Bertling W (1991): In situ hybridization studies on the expression of type X collagen in fetal human cartilage. Dev Biol 148:562–572.

Reichenberger E, Beier F, Lu Valle P, Olsen BR, von der Mark K, Bertling WM (1992): Genomic organization and full-length cDNA sequence of human collagen X. FEBS Lett 311:305–310.

Rosenthal N (1987): Identification of regulatory elements of cloned genes with functional assays. Meth Enzym 152: 704–720.

Rossert J, Eberspaecher H, de Crombrugghe B (1995): Separate cis-acting DNA elements of the mouse pro-α1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. J Cell Biol 129:1421–1432.

Rossouw CMS, Vergeer WP, du Plooy SJ, Bernard MP, Ramirez F, de Wet WJ (1987): DNA sequences in the first intron of the human pro-α_1(I) collagen enhance transcription. J Biol Chem 262:15151–15157.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning. A Laboratory Manual," 2nd ed. New York: Cold Spring Harbor Laboratory Press.

Schmid TM, Linsenmayer TF (1985a): Developmental acquisation of type X collagen in the embryonic chicken tibiotarsus. Dev Biol 107:373–381.

Schmid TM, Linsenmayer TF (1985b): Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. J Cell Biol 100:598–605.

Sokolov BP, Ala-Kokko L, Dhulipala R, Arita M, Khillan JS, Prockop DJ (1995): Tissue-specific expression of the gene for type I procollagen (COL1A1) in transgenic mice. Only 476 base pairs of the promoter are required if collagen genes are used as reporters. J Biol Chem 270:9622–9629.

Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ (1995): Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem 270: 9420–9428.

Thomas JT, Boot-Handford RP, Grant ME (1990): Modulation of type X collagen gene expression by calcium β-glycerophosphate and levamisole: Implications for a possible role for type X collagen in endochondral bone formation. J Cell Sci 95:639–648.

Thomas JT, Cresswell CJ, Rash B, Nicolai H, Jones T, Solomon E, Grant ME, Boot-Handford RP (1991a): The human collagen X gene. Complete primary translated sequence and chromosomal localization. Biochem J 280: 617–623.

Thomas JT, Kwan APL, Grant ME, Boot-Handford RP (1991b): Isolation of cDNAs encoding the complete sequence of bovine type X collagen. Biochem J 273:141–148.

Thomas JT, Sweetman WA, Cresswell CJ, Wallis GA, Grant ME, Boot-Handford RP (1995): Sequence comparison of three mammalian type-X collagen promoters and preliminary functional analysis of the human promoter. Gene 160:291–296.

von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, Stöss H (1992): Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 35:806–811.

von der Mark K, Frischholz S, Aigner T, Beier F, Belke J, Erdmann S, Burkhardt H (1995): Upregulation of type X collagen expression in osteoarthritic cartilage. Acta Orthop Scand S266:125–129.

Walker GD, Fischer M, Gannon J, Thompson RC, Oegema TRJ (1995): Expression of type X collagen in osteoarthritis. J Orthop Res 13:4–12.

Wang Z-Q, Grigoriadis AE, Wagner EF (1993): Stable murine chondrogenic cell lines from c-fos–induced cartilage tumors. J Bone Miner Res 8:839–847.

Yamaguchi N, Mayne R, Ninomiya Y (1991): The α_1(VIII) collagen gene is homologous to the α_1(X) collagen gene and contains a large exon encoding the entire triple helical domain and carboxy-terminal non-triple helical domains of the α1(VIII) polypeptide. J Biol Chem 266: 4508–4513.

Zhou G, Garofalo S, Mukhopadhyay K, Lefebvre V, Smith CN, Eberspaecher H, de Crombrugghe B (1995): A 182 bp fragment of the mouse pro-α_1(II) collagen gene is sufficient to direct chondrocyte expression in transgenic mice. J Cell Sci 108:3677–3684.