# An enhancer complex confers both high-level and cell-specific expression of the human type X collagen gene

David Chambers[1], David A. Young[2], Charles Howard, J. Terrig Thomas[3], David S. Boam, Michael E. Grant, Gillian A. Wallis, Raymond P. Boot-Handford*

*Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, 2.205 Stopford Building, Oxford Road, University of Manchester, Manchester M13 9PT, UK*

Received 11 July 2002; revised 8 October 2002; accepted 11 October 2002

First published online 29 October 2002

Edited by Takashi Gojobori

**Abstract Type X collagen expression is restricted to hypertrophic chondrocytes in the endochondral growth plate. Transient transfection of reporter constructs containing the human collagen X promoter into primary growth plate chondrocytes identified a *cis*-acting positive regulatory DNA element(s) that has cell-specific enhancer properties and binds a nuclear protein expressed specifically in growth plate chondrocytes. Functional disruption of this region results in a significant reduction in the activation of reporter gene transcription. The identified enhancer is a major element controlling both high-level and cell-specific expression of type X collagen gene.**


*Key words:* Chondrocytes; Type X collagen; Promoter; Enhancer

## 1. Introduction

During endochondral ossification, growth plate chondrocytes go through several stages of differentiation, including proliferation, maturation, and hypertrophy (e.g. see [1]). Type X collagen expression is restricted almost exclusively to hypertrophic chondrocytes [2], and the protein, a major product of these cells, appears to assemble into a hexagonal lattice similar to that formed by type VIII collagen [3]. Although the exact function of the collagen X gene remains to be determined, mutations found predominantly in the carboxyl-terminal non-collagenous domain of protein cause the human metaphyseal chondrodysplasia, type Schmid [4–6]. In situ hybridization and nuclear run-off studies have shown that type X collagen expression by hypertrophic chondrocytes is controlled at the transcriptional rather than translational level [7,8]. The mechanisms responsible for the tightly regulated expression of mammalian collagen X genes have been only partially characterized, although the presence of both positive [9–11] and negative [11,12] regulatory elements has been implicated. Here we show for the first time that a novel cell-specific enhancer element is responsible for controlling the highly regulated expression pattern of collagen X.

## 2. Materials and methods

### 2.1. Promoter constructs

Promoter fragments for use in expression constructs were generated by either restriction enzyme digestion or PCR from the human lambda genomic clone [HX3] [9]. pCAT Promoter and enhancer reporter vectors as described were obtained from Promega. Transfection grade DNA was prepared using a double cesium chloride banding method [13] or the Qiagen column purification technique. The concentration and purity of the plasmid preparations were determined spectrophotometrically at 260/280 nm [13].

### 2.2. Cell culture

Growth media and supplements for cell culture were obtained from Gibco BRL. Bacterial collagenase (type 1A) was obtained from Sigma Chemicals. Bovine fetal calves (second trimester [14]) were obtained from a local abattoir and the long bones used as a source of growth plate and epiphyseal chondrocytes as described previously [9]. Chondrocytes were plated at $1\times10^6$ cells/cm$^2$ in NUNC tissue culture dishes (9 cm$^2$) and maintained in DMEM buffered to pH 7.2 with 10 mM HEPES and containing 0.37% (w/v) sodium bicarbonate, penicillin (100 units/ml), streptomycin (100 mg/ml), 2 mM glutamine and 10% (v/v) fetal calf serum (FCS). The medium was replaced on alternate days and all cultures were maintained at 37°C in a 5%:95% (v/v) $CO_2$:air atmosphere. RT-PCR was used to confirm the expression of collagen X in growth plate but not in epiphyseal chondrocytes.

Normal human skin fibroblasts and HeLa cells were maintained in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), gentamicin (10 mg/ml), 2 mM glutamine, and 5% (v/v) fetal calf serum. For transfection purposes fibroblasts and HeLa cells were plated into 9-cm$^2$ dishes at a density of $2.5\times10^5$ cells/dish on the day before transfection. The growth medium was replaced on the following morning, at least 2 h before transfection.

### 2.3. Transfection and CAT assays

Transient transfections were performed by calcium phosphate co-precipitation [9]. Growth plate and epiphyseal chondrocyte preparations were treated with bovine testes hyaluronidase (4 units/ml) (Sigma) for 24 h prior to and for the first 4 h following transfection [15]. Each dish of cells was co-transfected with 20 μg of the collagen X promoter CAT vector and 3 μg of a plasmid containing the human growth hormone (hGH) gene driven by a CMV promoter (Hybridtech). Four hours post-transfection, cells were shocked by the addition of 20% (v/v) DMSO (Sigma) in PBS (without $Mg^{2+}$ and $Ca^{2+}$) for 1 min. The cell layer was then washed twice with PBS (without $Mg^{2+}$ and $Ca^{2+}$) and the growth medium replaced. Forty-eight hours post-transfection, the growth medium was aspirated and used for assay of

*Corresponding author. Fax: (44)-161-275-5082.
*E-mail address:* ray.boot-handford@man.ac.uk (R.P. Boot-Handford).

[1] Present address: Department of Developmental Neurobiology, 4th Floor New Hunts House, Guy's Campus, King's College, London SE1 1UL, UK.
[2] Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK.
[3] Present address: Bone Research Branch, NIDR, NIH, Bethesda, MD 20892, USA.

0014-5793/02/$22.00 
PII: S0014-5793(02)03606-2

hGH (Tandem-R hGH ImmunoRadioMetric assay kit, Hybridtech). Cell layers were suspended in 150 μl of 0.25 M Tris–HCl pH 8.0 and extracts prepared by three rounds of freezing in dry ice–ethanol/thawing at 37°C, followed by vigorous vortexing. Cell debris was removed by microfuging (10 min), the cell extract heated to 68°C for 10 min to inactivate endogenous acetylases and stored at −70°C.

CAT assays were performed on cell extracts (50 μl) as described previously [16]. Variations in transfection efficiencies were standardized using the data from the human growth hormone assay. All values are given as mean ± S.E.M. ($n = 6$ unless otherwise stated).

### 2.4. Preparation of nuclear extracts

Nuclear extracts from collagen X-expressing hypertrophic chondrocytes were prepared [17] and mobility shift assays on $^{32}$P-labeled enhancer-derived fragments performed as previously described [18].

## 3. Results and discussion

### 3.1. Identification of an upstream positive regulatory element

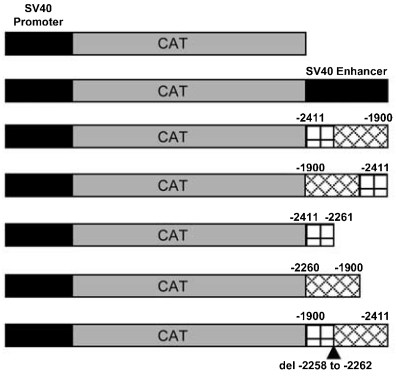
We have previously shown that nucleotides −2411 to +34 of the type X collagen promoter directed strong CAT expression in the growth plate chondrocytes but weak or no expression in epiphyseal chondrocytes or fibroblasts [9]. By examining a more detailed series of deletion constructs, a region from −2411 to −1900 was found to be essential for the high-level and cell-specific expression (data not shown). A major reduction in CAT activity (99% ± 5% to 4% ± 1%) was noted when the sequences from −2411 to −1900 were deleted, clearly indicative of the presence of a strong cell-specific element.

### 3.2. The upstream positive regulatory element behaves as an enhancer

The potential enhancer activity of the −2411 to −1900 human COL10A1 promoter element was tested by inserting the fragment into the pCAT promoter vector which contains the SV40 promoter, but lacks an enhancer (Fig. 1). The pCAT promoter vector alone gave no detectable activity in either growth plate chondrocytes or fibroblasts whereas the pCAT control vector containing both the SV40 promoter and enhancer produced high-level expression in both cell types (Fig. 1). The pCAT promoter vector containing the −2411 to −1900 fragment of the COL10A1 promoter supported high-level CAT expression in growth plate chondrocytes in both the positive (90% ± 5%) and the negative (70% ± 3%) orientation. This effect is cell-specific since the same constructs gave no expression in fibroblasts (Fig. 1). A similar enhancing effect was observed when the 512-bp fragment was placed upstream of a HSV TK promoter driving a CAT reporter gene (data not shown). To more finely map the location of the enhancer, the 512-bp insert in the pCAT-Pro-512+ve plasmid was subdivided into two smaller fragments by restriction digestion with *Spe*I: one of 151 bp (−2411 to −2261) and the 3-prime 361 bp (−2260 to −1900). However, neither the pCATPro-151 nor the pCAT-Pro-361 plasmid induced CAT expression in growth plate chondrocytes (Fig. 1). In addition, similar patterns of CAT activity were observed upon transfection of the promoter constructs either in the absence of the hGH transfection control plasmid or when using liposome-mediated transfection protocols (data not shown).

### 3.3. The enhancer element is recognized by a growth plate specific factor

One explanation for the loss of the enhancer activity observed upon subdivision of the 512-bp fragment at the *Spe*I site is that the *cis* regulatory element is located in the vicinity of the *Spe*I site at base −2260 in the distal promoter. We therefore generated a double-stranded oligomer containing sequences from −2273 to −2244 (5′-TAAAGCCACAGACTAGTCACTTCAAACAGC-3′ [*Spe*I site underlined]) to determine by band shift analyses whether a nuclear protein from growth plate chondrocytes bound this region. Growth plate chondrocyte-derived nuclear extract with the $^{32}$P-labeled 30-bp enhancer-derived fragment gave a single specific gel-retarded band compared to labeled probe alone (Fig. 2B, lane 2 vs. lane 1, respectively). This interaction was shown to be specific since a 500-fold excess of unlabeled fragment completely abolished binding of the growth plate chondrocyte




B

| | Growth plate chondrocytes | Fibroblasts |
|---|---|---|
| pCAT-Promoter | <1 | <1 |
| pCAT-Control | 100±3 | 95±3 |
| pCAT-Pro-512+ve | 90±5 | <1 |
| pCAT-Pro-512-ve | 70±3 | <1 |
| pCAT-Pro-151+ve | <1 | <1 |
| pCAT-Pro-361+ve | <1 | <1 |
| pCAT-Pro-507-ve | 42±4 | <1 |

Fig. 1. Localization and disruption of a cell-specific enhancer element in the distal promoter of the COL10A1 gene. A: To examine the cell specificity of the upstream *cis*-acting positive regulatory elements, short fragments of the distal promoter were prepared and cloned into the enhancerless CAT vector containing the SV40 heterologous promoter (pCAT promoter). Constructs were prepared as described in Section 2. The small black box represents the SV40 promoter element, the larger black box the SV40 enhancer, and the hatched box the human distal promoter region from between −2411 and −1900. B: Values (mean ± S.E.M., $n = 6$) were standardized for transfection efficiency using the results from the human growth hormone assay as described in Section 2. Data are expressed relative to the CAT activity in type X collagen-expressing cells transfected with pCAT control (defined as 100%).

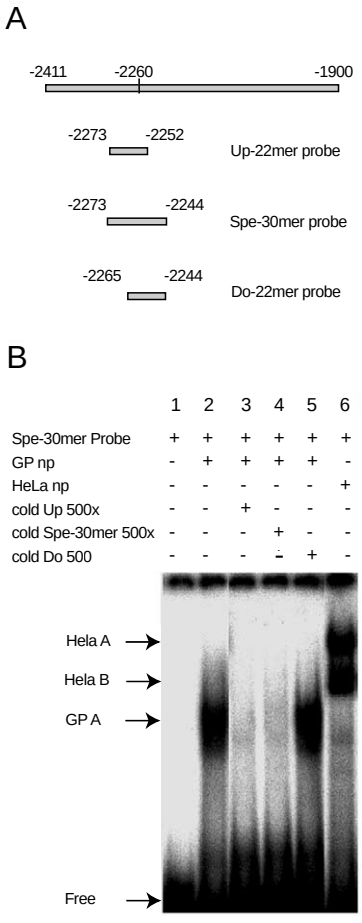



Fig. 2. A 30-bp fragment in the distal promoter is bound by a specific *trans*-acting factor in growth plate chondrocytes. A: A schematic diagram of the 512-bp human collagen X gene enhancer fragment. Probes for band shift studies were localized around the position of a *Spe*I restriction endonuclease site at −2260. B: Electrophoresis mobility shift assay using a $^{32}$P-labeled double-stranded DNA fragment representing nucleotides of −2273 to −2244 (Spe-30mer Probe) of the human collagen X gene promoter and nuclear precipitates (np) from either collagen X-expressing growth plate (GP) chondrocytes or negative control HeLa cells. The electrophoretic position of the free $^{32}$P-labeled probe is shown in lane 1 and indicated by the arrow labeled 'Free'. The presence of a single gel-retarded band after incubation with growth plate nuclear precipitates is indicated by the arrow marked GP A in lane 2. The interaction of the Spe-30mer Probe with this protein can be completely abolished by incubation with 500-fold excess of unlabeled Spe-30mer Probe (lane 4) or an unlabeled double-stranded DNA fragment constituting nucleotides −2273 to −2252 (Up, lane 3). The retarded band observed from growth plate nuclear precipitates cannot be competed for by a 500-fold excess of an unlabeled DNA segment constituting nucleotides −2266 to −2244 (Do, lane 5). In cells that do not express the collagen X gene, the 30-bp fragment produces two distinct mobility-shifted bands (lane 6) labeled HeLa and HeLa B, respectively.

nuclear protein to the labeled 30-bp fragment (Fig. 2B, lane 4). Using the same $^{32}$P-labeled 30-bp enhancer-derived fragment with HeLa cell-derived nuclear extract (-ve control), a different retardation profile consisting of two distinct mobility-shifted bands was observed (Fig. 2B, lane 6). To further define the sequence recognized by the putative positive regulatory factor(s) contained within the growth plate chondrocyte nuclear extract, additional competition experiments were performed with sub-fragments of the 30-bp sequence. The binding of the growth plate chondrocyte-specific protein to the $^{32}$P-labeled 30-bp enhancer fragment could be completely ablated by introducing a 500-fold excess of unlabeled competitor fragment which constituted the most 5′ 22 bases of the 30-bp enhancer domain (Up; −2273 to −2252: 5′-TAAAGCCA-CAGACTAGTCACTT-3′; Fig. 2B, lane 3). In contrast, the most 3′ 22 bases (Do) of the 30-bp enhancer failed to interfere with protein binding and subsequent gel retardation (Fig. 2B, lane 5). Taken together, these data show that the putative positive *trans*-acting factor binds within a specific 22-bp sequence located between −2273 and −2252 in the collagen X gene promoter/enhancer region that includes the *Spe*I site at −2260. Examination of the entire sequence comprising the 30-bp enhancer using a DNA sequence analysis package (http://transfac.gbf.de/cgi-bin/patSearch/patsearch.pl/) failed to identify any known positive or negative regulating transcription factor recognition motifs, suggesting that the cell-specific factor binding this sequence is novel.

### 3.4. Sequences around −2260 direct high-level CAT activity in chondrocytes

The 30-bp fragment (−2273 to −2244) that binds a specific protein expressed in growth plate chondrocytes was tested to see if it could drive cell-specific transcription from a heterologous promoter. Neither a single copy of the fragment, nor three concatenated copies placed upstream of the TK promoter driving a CAT gene produced detectable transcription in growth plate chondrocytes, although transfection was successful based on hGH assays and the CAT activities produced by the positive controls (data not shown). Thus the 30-bp region of the promoter located between −2273 and −2244 appears necessary but not sufficient for the activation of the collagen X gene in hypertrophic chondrocytes. However, the importance of the 30-bp region's contribution to promoter activation was confirmed when the deletion of five nucleotides of the *Spe*I site (pCAT-Pro-507-ve, bases −2262 to −2258) in the 512-bp enhancer fragment reduced reporter gene activity from the control (pCAT-Pro-512-ve, see Fig. 1) level of 100% ± 10% ($n$ = 3) to 42% ± 4% ($n$ = 6) in growth plate chondrocytes.

### 3.5. Interpretation of the collagen X promoter analysis

We present clear evidence that the sequences from −2411 to −1900 of the COL10A1 gene contain a strong enhancer element capable of driving high-level CAT expression in a growth plate chondrocyte specific fashion (Fig. 1). The enhancer activity is completely lost if the 512-bp enhancer fragment is cut into two (151 and 361 bp, respectively) at the internal *Spe*I site, neither fragment being able to enhance any cell-specific transcription (Fig. 1). One explanation for the complete loss of enhancer activity upon *Spe*I cleavage of the active 512-bp fragment is that the enhancer element includes the *Spe*I site as part of the DNA binding site for the transcription/enhancer complex. Indeed, the 30-bp sequence flanking and including the *Spe*I site binds a transcription complex present in the nuclei of growth plate chondrocytes but not in HeLa cells (Fig. 2). If the identified transcription complex is the sole factor responsible for generating the detected enhancer activity, the 30-bp sequence containing the binding site would be able to substitute completely for the 512-bp enhancer element and drive expression specifically in growth

plate chondrocytes. However, we demonstrated that neither a single nor multiple copies of the 30-bp fragment is capable of producing enhancer activity, suggesting that this element must be acting in concert with another located elsewhere in the 512-bp fragment. Since it is clear that neither sequences 5′ nor 3′ of the *Spe*I site can generate enhancer activity in isolation, we ablated the *Spe*I sequence within the 512-bp enhancer fragment, anticipating that this would abolish enhancer activity. However, activity of the resulting construct was not abolished but, instead, significantly decreased (pCAT-pro-507-ve, Fig. 1). Therefore, further elements located both upstream and downstream of the *Spe*I site must be present and cooperate to produce the full strength enhancer activity exhibited by the 512-bp fragment. Interestingly, none of these sites is capable of producing enhancer activity in isolation. These data are the first to demonstrate the presence of a complex enhancer element that appears both necessary and sufficient for high-level and cell-specific expression of the human collagen X gene. Whilst the enhancer element contained within the 512-bp fragment is sufficient to produce both high-level and cell-specific expression of the collagen X gene, other factors located nearer the transcription start site clearly modulate or fine tune the gene's expression. For instance, sequences between positions −452 and +1 control responsiveness to c-Raf [12], osteogenic protein-1 (OP-1) or BMP7 [19]. In addition, recent data has shown that the −1870 to −2407 region of the human collagen X promoter is involved in the negative regulation of transcription in response to parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) [20]. Thus, it may be that the negative regulatory PTH/PTHrP response element interacts either competitively or cooperatively with the *trans*-acting factors that bind to the enhancer element described here to regulate transcription. To date, the transcription factors regulating the enhancer activity of the human collagen X gene have not been identified. Further analyses of the −2411 to −1900 region of the promoter will enable the identification of the other sequences required for the full enhancer activity and the characterization of the *trans*-acting factors involved.

*Acknowledgements:* This work was supported by the UK Arthritis Research Campaign.

## References

[1] Chen, Q., Johnson, D.M., Haudenschild, D.R. and Goetinck, P.F. (1995) Dev. Biol. 172, 293–306.
[2] Gibson, G.J., Schor, S.L. and Grant, M.E. (1982) J. Cell Biol. 93, 767–774.
[3] Kwan, A.P.L., Cummings, C.E., Chapman, J.A. and Grant, M.E. (1991) J. Cell Biol. 109, 1849–1856.
[4] Warman, M.L., Abbott, M., Apte, S.S., Hefferon, T., McIntosh, I., Cohn, D.H., Hecht, J.T., Olsen, B.R. and Francomano, C.A. (1993) Nat. Genet. 5, 79–82.
[5] Wallis, G.A., Rash, B., Sweetman, W., Thomas, J.T., Super, M., Evans, G., Grant, M.E. and Boot-Handford, R.P. (1994) Am. J. Hum. Genet. 54, 169–178.
[6] Wallis, G.A., Rash, B., Sykes, B., Bonaventure, J., Maroteaux, P., Zabel, B., Wynne-Davies, R., Grant, M.E. and Boot-Handford, R.P. (1996) J. Med. Genet. 33, 450–457.
[7] Linsenmayer, T.F., Chen, Q.A., Gibney, E., Gordon, M.K., Merchant, J.K., Mayne, R. and Schmid, T.M. (1991) Development 111, 191–196.
[8] LuValle, P., Daniels, K., Hay, E.D. and Olsen, B.R. (1992) Matrix 12, 404–413.
[9] Thomas, J.T., Sweetman, W.A., Cresswell, C.J., Wallis, G.A., Grant, M.E. and Boot-Handford, R.P. (1995) Gene 160, 291–296.
[10] Chambers, D., Thomas, J.T., Boam, D.S., Wallis, G.A., Grant, M.E. and Boot-Handford, R.P. (1996) Ann. New York Acad. Sci. 785, 227–230.
[11] Beier, F., Vornehm, S., Poschl, E., von der Mark, K. and Lammi, M.J. (1997) J. Cell. Biochem. 66, 210–218.
[12] Beier, F., Taylor, A.C. and LuValle, P. (1999) J. Cell. Biochem. 15, 549–557.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[14] Bogart, R. (1959) Improvement of Livestock, 316 pp., Macmillan Press, New York.
[15] LuValle, P., Iwamoto, M., Fanning, P., Pacifici, M. and Olsen, B.R. (1993) J. Cell Biol. 121, 1173–1179.
[16] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell Biol. 2, 1044–1051.
[17] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[18] Ausubel, F.M. Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current Protocols in Molecular Biology, pp. 12.2.1–12.2.7, John Wiley and Sons, New York.
[19] Harada, S., Sampath, T.K., Aubin, J.E. and Rodan, G.A. (1997) Mol. Endocrinol. 11, 1832–1845.
[20] Riemer, S., Gebhard, S., Beier, F., Poschl, E. and Von der Mark, K. (2002) J. Cell. Biochem. 86, 688–699.