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Role of c-fos in the Regulation of Type X Collagen Gene Expression by PTH and PTHrP: Localization of a PTH/PTHrP-Responsive Region in the Human COL10A1 Enhancer

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Abstract PTH and PTHrP have been shown to inhibit maturation of growth plate chondrocytes and the expression of type X collagen. In order to examine the regulatory mechanisms involved, fetal bovine growth plate chondrocytes were incubated for 24–48 h under serum-free conditions with PTH and PTHrP and various aminoterminal, midregional, and carboxyterminal fragments of these hormones. Analysis of type X collagen mRNA levels by Northern hybridization showed a significant suppression by PTH (1–84), PTH (1–34), and PTHrP (1–40), but not by PTH (28–48) or PTH (53–84). PTH fragment (3–34) did not reduce α1(X) mRNA levels, while *bis*-indolylmaleimide, an inhibitor of the protein-kinase C pathway, did not affect α1(X) mRNA suppression by PTH, supporting the notion that the inhibition of type X collagen expression by PTH involves predominantly the adenylate cyclase pathway of the PTH/PTHrP-receptor. Since PTH and PTHrP have been shown to induce c-fos in osteoblasts and chondrocytes, the possibility was tested that c-fos mediated the suppressive effect of PTH/PTHrP on collagen X expression. In fetal bovine hypertrophic chondrocytes PTH (1–34), but not PTH (3–34) nor the midregional or C-terminal PTH fragments induced c-fos expression. In order to identify *cis*- and *trans*-acting elements in the COL10A1 gene involved in c-fos-mediated inhibition of collagen X expression by PTH/PTHrP, reporter gene constructs carrying various fragments of the human COL10A1 promoter coupled to the luciferase gene were transfected into hypertrophic chondrocytes. A tissue-specific, strong enhancer region, which we had previously located in the promoter of the human type X collagen gene COL10A1, was further narrowed down to a 530-bp sequence, located between − 1,870- and − 2,407 bp upstream of the transcription start site. The transcriptional activity of this enhancer element in transfected hypertrophic chondrocytes was significantly reduced after incubation with PTH (1–34) or PTHrP (1–40). Transcription of these reporter genes was also inhibited when chondrocytes were cotransfected with a c-fos expression vector. These results indicate the presence of a PTH/PTHrP responsive element in the human COL10A1 enhancer, which may be represented by multiple putative AP-1 sites located in this region. J. Cell. Biochem. 86: 688–699, 2002.

Key words: parathyroid; c-fos; chondrocyte; collagen type X; enhancer; transcription

Several in vivo and in vitro studies have shown that both parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) enhance survival of pre-hypertrophic growth plate chondrocytes, stimulate their proliferation, and prevent or delay differentiation to hypertrophic chondrocytes [Amizuka et al., 1994; Iwamoto et al., 1994; Henderson et al., 1996; Lee et al., 1996; Weir et al., 1996]. In vitro, the aminoterminal fragments PTH (1–34) and PTHrP (1–40) stimulate ^{3}H-thymidine incorporation and proliferation of fetal chondrocytes [Koike et al., 1990; Henderson et al., 1996] and suppress the synthesis of type X collagen, a marker of chondrocyte hypertrophy. This was shown in long-term rabbit growth plate chondrocyte cultures [Iwamoto et al., 1994] and in freshly-isolated chick growth plate

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chondrocytes [O'Keefe et al., 1997]. These in vitro findings are corroborated by studies on the role of PTHrP and the PTH/PTHrP-receptor in endochondral ossification in transgenic mice: Mice deficient in both alleles for PTHrP show a markedly reduced number of proliferating chondrocytes in the growth plate, premature chondrocyte hypertrophy and a severely disturbed zone of endochondral ossification [Amizuka et al., 1996; Karaplis and Kronenberg, 1996; Lee et al., 1996]. Similarly, PTH/PTHrP receptor-deficient mice exhibited accelerated differentiation of growth plate chondrocytes [Lanske et al., 1996]. Constitutive or targeted overexpression of PTHrP in cartilage, on the other hand, caused a delay in chondrocyte hypertrophy and endochondral ossification, resulting in chondrodysplasia [Henderson et al., 1996; Weir et al., 1996; Schipani et al., 1997].

These studies as well as most previous cell culture studies on the role of PTH/PTHrP on growth plate chondrocytes were long-term studies in which type X collagen was used as an end point marker of chondrocyte hypertrophy. In recent studies [Erdmann et al., 1996; O'Keefe et al., 1997; Grimsrud et al., 1998], immediate effects of PTH and PTHrP on collagen X expression by freshly-isolated growth plate chondrocytes were observed within 24–48 h both at protein and mRNA level. Little is known, however, on the mechanism of PTH/PTHrP induced inhibition of collagen X gene expression. Previously, we and others have described a tissue-specific, strong enhancer region in the promoter of the human type X collagen gene (COL10A1), which stimulated transcriptional activity of luciferase reporter genes containing various fragments of the human COL10A1 promoter after transfection into hypertrophic bovine chondrocytes [Chambers et al., 1996; Beier et al., 1997]. Here, we localize the enhancer activity to the region between $-1{,}870$- and $-2{,}407$-bp upstream of the transcription start site and show that PTH (1–34) and PTHrP (1–40) at 10^{-8} M profoundly downregulate transcription of reporter gene constructs contain this enhancer element.

Several lines of evidence suggest participation of c-fos in PTHrP-induced inhibition of collagen X gene expression: PTHrP has been shown to induce c-fos in osteoblasts [Kano et al., 1994; Lee et al., 1994; McCauley et al., 1997] and in hypertrophic chondrocytes [Lee et al., 1994; Ionescu et al., 2001]. Furthermore, c-fos-deficient mice exhibit a similar phenotype in the growth plate [Wang et al., 1992] as the PTH/PTHrP receptor knockout mice; finally, c-fos overexpression inhibits chondrocyte differentiation in vitro [Thomas et al., 2000].

In order to elucidate the mechanism of PTH/PTHrP-induced inhibition of collagen X gene expression and the role of c-fos in this process, we investigated the effect of aminoterminal, midregional, and carboxyterminal fragments of PTH and PTHrP, which activate different PTH/PTHrP receptor pathways on collagen X expression in fetal bovine growth plate chondrocytes. We show that predominantly the aminoterminal region of PTH/PTHrP is responsible for the inhibitory effect, indicating the involvement of the adenylate cyclase/PKA pathway. These PTH/PTHrP fragments also induced c-fos expression in hypertrophic, but not in resting chondrocytes. Co-expression of c-fos in hypertrophic chondrocytes suppressed transcriptional activity of reporter genes containing the collagen X enhancer element. This indicates that the inhibitory effect of PTH/PTHrP on collagen X gene expression is mediated by c-fos, which may interact indirectly or directly by binding to the multiple putative AP-1 sites located in the enhancer.

MATERIALS AND METHODS

Chemicals and Supplies

Bovine (b) and human (h) PTH fragments were obtained from Sigma (Deisenhofen, Germany): full-length hPTH (1–84), bPTH (1–34), $NLC^{8,18}$, Tyr^{34} bPTH (3–34), hPTH (28–48), hPTH (52–84) PTHrP (1–40). DMEM/F12 cell culture medium, fetal calf serum (FCS) (heat-inactivated) and cell culture reagents, including sodium pyruvate (UPS grade) were from Life Technologies GmbH (Eggenstein, Germany). Testicular hyaluronidase and clostridial collagenase P were from Boehringer-Roche (Germany). The luciferase reporter gene vector used for transfection experiments (pGL2 basic), the β-galactosidase reporter gene plasmid (pSVβ-gal) and the luciferase reporter gene assay system were obtained from Promega (Madison, WI). [Methyl-^{3}H]-thymidine (37 MBq/ml) was purchased from Amersham-Buchler Gmbh (Germany). Colchicine and bisindolylmaleimide I were from Sigma-Aldrich GmbH.

Cell Culture

Chondrocytes from the growth plate of fetal calves (vertex-breech length: 40–50 cm, around second trimester of gestation) were prepared as described previously [Erdmann et al., 1996], with slight modification: The washed growth plates were digested with 0.04% clostridial collagenase P (Roche/Boehringer Mannheim, Germany) in DMEM/F12 for 12 h at 37°C under gentle shaking, normally in the presence of 5% FCS. Chondrocytes were filtered though a 40-μm cell strainer, counted, washed with DMEM/F12 without serum, and kept serum free in standard culture medium (DMEM/F12 1:1, containing penicillin/streptomycin, 1-mM pyruvate, 1-mM L-cysteine, 50 μg/ml L-(+) ascorbate). Incubation with PTH peptides was done for 24 or 48 h in monolayer culture dishes in standard medium at cell densities of 10^5 cells/cm^2. For transient transfection studies with reporter genes, 10^6 chondrocytes were seeded into six-well plates (well diameter 3.5 cm) in standard medium containing 5% FCS and 20 U/ml testicular hyaluronidase and allowed to adhere for 1–2 h before transfection.

Northern Hybridization

RNA was extracted from chondrocytes with 4-M guanidinium isothiocyanate according to Chirgwin et al. [1979] and purified by acid phenol extraction according to Chomczynski and Sacchi [1987] using the RNAid™ plus kit (Biomol, La Jolla, CA). The purified total RNA was subjected to agarose gel electrophoresis (10 μg/lane) in denaturing formaldehyde gels, blotted onto nylon filters, cross-linked by UV, and hybridized at 43°C in 50% formamide to collagen cDNA probes as described [Erdmann et al., 1996]. A 2.2-kb cDNA probe coding for most of the third exon of COL10A1 was excised from the plasmid SX2200 [Beier et al., 1997] with SacI and XhoI, and labeled with ^{32}P-dCTP by random priming as described [Erdmann et al., 1996]. For the analysis of COL2A1 transcripts a 1.4-kb cDNA probe coding for the untranslated 3′ end of COL2A1 was excised from the plasmid pHCAR3 [Aigner et al., 1993]. For detection of c-fos a 1.0-kb fragment of human c-fos cDNA was used. A 1.3-kb fragment of GAPDH cDNA probe was used for standardization. The hybridized and washed filters were exposed to Hyperfilm-MT autoradiography films (Amersham-Buchler GmbH) at −70°C. For quantitative evaluation, bound radioactivity was measured with a phosphorimager (Fujifilm BAS-1500 (Fuji, Japan) and the accessory software program TINA2.08.

Overexpression of c-fos

For overexpression of c-fos in growth plate chondrocytes, cells were transfected with 10 μg of either pRSV-cfos expression vector (kindly provided by Dr. P. Angel, DKFZ, Heidelberg) or RSV-0 as control vector using Fugene. After 48 h cells were lyzed, and α1(X) mRNA levels were determined by Northern hybridization. In transient transfection studies with reporter gene constructs, chondrocytes were cotransfected with 4 μg of pGL basic or the COL10A1 promoter constructs as described below.

Reporter Gene Plasmids

Luciferase reporter gene plasmids were constructed by inserting various fragments of the human COL10A1-promoter together with 31 nucleotides from the first exon into the vector pGL2 basic [Beier et al., 1997]. The following plasmids were used in this experiment: pGL3000 spanning a fragment of −2,864 to +31 bp relative to the transcription start site of COL10A1, pGL2500 −2,407 to +31 bp), pGL 900 (−868 to +31) and pGL500 (−460 to +31) [Beier et al., 1997]; pGL3000 contained an additional putative silencer element between −2,407 and −2,861 bp described previously [Beier et al., 1997]. In pGL 900-1/8, the 530-bp enhancer element located between −1,870 and −2,407 bp was coupled to pGL 900, while in the pGL900-530 construct, the enhancer element was placed 3′ of the luciferase gene in reverse direction (Fig. 1). In the pGL2500inverse and pGL3000inverse, the promoter sequences between −868 and −2,407 or 2,861 bp, respectively, were ligated to the 3′ end of pGL900.

Sequence Analysis for Putative AP-1 Sites

The human COL10A1 sequence was screened for putative AP-1 binding sites using the Mat inspector V2.2 program to browse the Transfac data base. From the generated matrix on sequences were considered as AP-1 sites, which contained T-(G/T)-A-S-T-(C/A)-A as core palindromic sequence [Risse et al., 1989].

Transient Transfections

Growth plate chondrocytes were cultured in six-well plates in standard medium containing

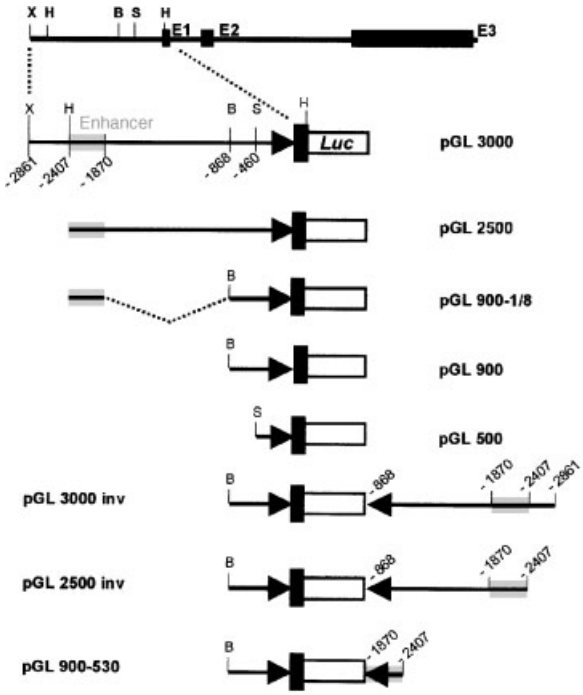

Fig. 1. Luciferase reporter gene constructs containing various fragments of the human COL10A1 promoter. Genomic fragments of the human COL10A1 promoter up to 3,000-bp upstream of the transcription start site including the 5′ part of the first exon were ligated to the luciferase gene as described in Materials and Methods. In the pGL900-1/8 construct, the 530-bp enhancer element was ligated directly to the 900-bp basal promotor; in pGL2500 inv, pGL3000inv, and pGL 900-530, the promoter sequences were ligated in reverse direction to the 3′ end of the luciferase gene in order to examine position- and orientation-independent activity of the enhancer.

5% FCS for 1–2 h and cotransfected for 6 h at 37°C in 5% CO_2 with 10-μg COL10A1 luciferase reporter gene constructs and 4 μg of the plasmid pSVß-Gal (Promega), precipitated with Ca-phosphate. To enhance transfection efficiency, 20 U/ml testicular hyaluronidase were added [Luvalle et al., 1993; Beier et al., 1997]. Cells were washed, and then incubated in serum-free medium for 24 or 48 h in the presence of 10^{-8} M PTH or PTHrP peptides. Cells were lysed, and the cytosolic luciferase was analyzed according to the manufacturer's protocol (Promega). β-galactosidase activity was determined by incubating the cell lysate with ONPG substrate for 4 h and colorimetric analysis. Relative transcriptional activity was then calculated presenting the ratio of luciferase activity vs. the normalized galactosidase activity. Luciferase and galactosidase data represent average values and standard deviations of triplicate cell culture wells of three independent experiments each, using three different chondrocyte populations.

RESULTS

Effect of PTH and PTHrP Peptides on Collagen X mRNA Levels

PTH, PTHrP, and their aminoterminal fragments PTH (1–34) and PTHrP (1–40) suppress at 10^{-8} Mα1(X) mRNA levels in fetal bovine growth plate chondrocytes by 60–90% after 48 h incubation under serum-free conditions (Figs. 2 and 4). Time course studies revealed that suppression of α1(X) mRNA levels by PTH (1–84) started already after 2 h (Fig. 3). The data also demonstrate a 40% decrease of α1(X) mRNA levels after 48 h in control cultures, in comparison to 80% decrease in the presence of PTH (1–34) or PTH (1–84) after 48 h. The experiments were performed in the presence of colchicine, an inhibitor of mitosis, in order to exclude the possibility that the relative loss of α1(X) mRNA compared to GAPDH standard was a result of overgrowth by proliferating, type X collagen-negative chondrocytes present in the

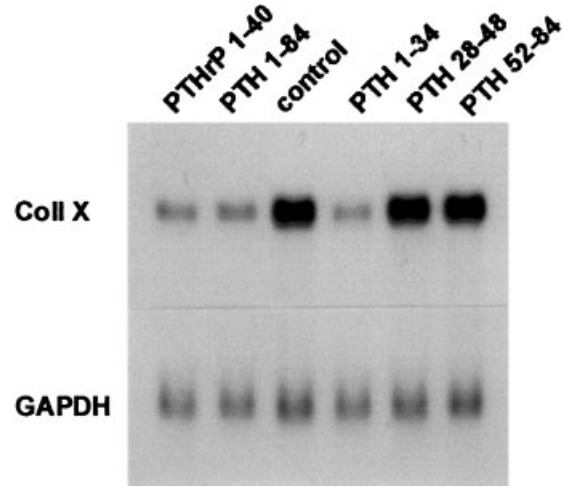

Fig. 2. Suppression of α1(X) mRNA levels in cultures of bovine growth plate chondrocytes by PTH, PTHrP, and PTH (1–34). Freshly-isolated hypertrophic chondrocytes were incubated with PTH peptides for 48 h at a final concentration of 10^{-8} M under serum-free conditions in the presence of colchicine (10^{-6} M) for 24 h to block mitosis. The α1(X) mRNA levels were determined by Northern hybridization, and the signals were measured using a phosphorimager and corrected for GAPDH signal intensity. In all experiments, PTH (1–84) and PTH (1–34) reduced the α1(X) and α1(II) mRNA levels significantly by 60–90%, while PTH (28–48) and PTH (52–84) had no or only minimal stimulatory effects.

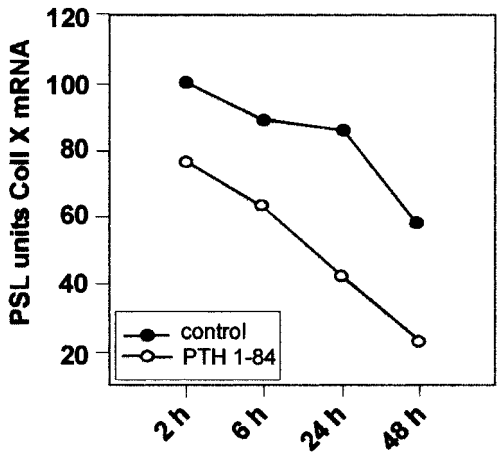

Fig. 3. Time course of α1(X) mRNA steady state levels of fetal bovine growth plate chondrocytes cultured in the presence or absence of PTH (1–84). Freshly-isolated cells were incubated with 10^{-8} M PTH (1–84) under serum-free conditions and harvested after 2, 6, 24, and 48 h for RNA isolation. The α1(X) mRNA levels as measured by Northern hybridization decline with time both in the absence and presence of PTH, but significantly faster in the presence of PTH.

growth plate chondrocyte population [Erdmann et al., 1996]. Thus, the results of this study proved that PTH, PTHrP (1–40), and PTH (1–34) inhibit type X collagen expression in the absence of mitosis (Fig. 5).

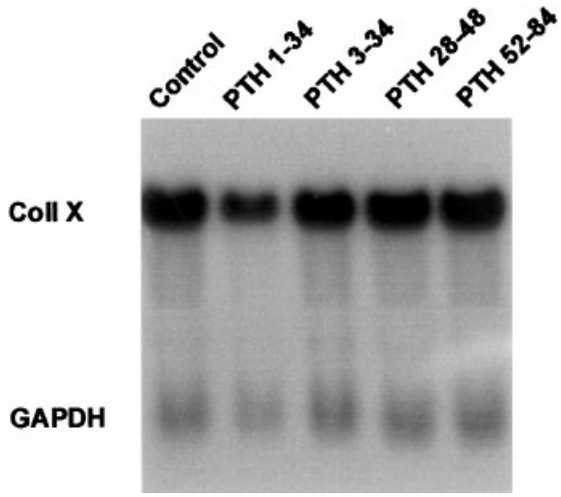

Fig. 4. The aminoterminal residues, 1–2, are essential for the suppressive effect of PTH. Bovine growth plate chondrocytes were incubated in the absence or presence of PTH (1–34), PTH (3–34), PTH (28–48), PTH (52–84) at 10^{-8} M, and the α1(X) mRNA levels were determined by Northern hybridization. The data show that PTH (3–34) does not suppress α1(X) mRNA levels, indicating that the aminoterminal residues of PTH(1–34) are required for the suppressive effect.

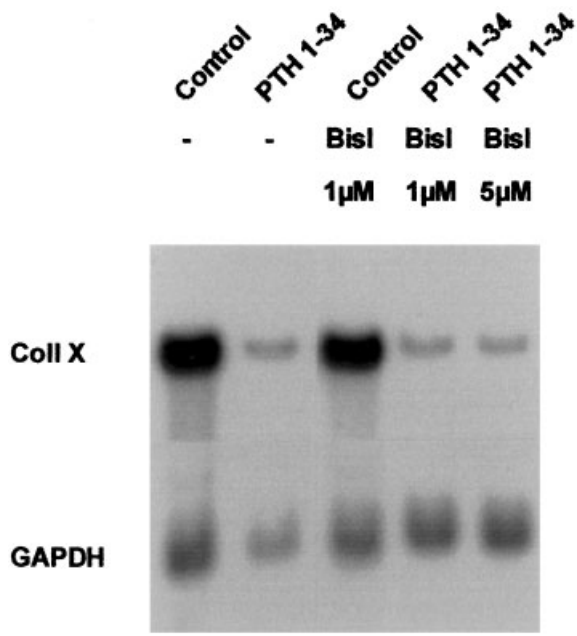

Fig. 5. The suppressive effect of PTH (1–34) does not involve protein kinase C. Bovine growth plate chondrocytes were incubated for 24 h in the absence or presence of PTH (1–34) at 10^{-8} M together with bisindolylmaleimide I (bis I) at 1 and 5 μM, which blocks PKC activity. α1(X) mRNA levels as determined by Northern hybridization by PTH (1–34) was unaffected, indicating that PKC activity is probably not involved.

Suppression of COL10A1 Expression by PTH (1–34) Involves the Adenylate Cyclase Pathway

In order to decide whether the suppression of COL10A1 expression by PTH and PTHrP involves the adenylate cyclase pathway/PKA pathway or the phospholipase C/proteinkinase C pathway, which are both activated by the PTH/PTHrP receptor [Abou-Samra et al., 1992; Jüppner, 1995; Kronenberg et al., 1998], cells were treated with PTH (3–34). Only a minimal inhibition of type X collagen expression was observed (Fig. 4), confirming that the suppression of type X collagen mRNA by PTH (1–34) involves predominantly the adenylate cyclase pathway, which is activated by the aminoterminal residues PTH (1–2) [Chakravarthy et al., 1990]. In corroboration with this result, inhibition of the protein kinase C (PKC) activity with *bis*-indolylmaleimide at 1 and 5 μM did not affect the PTH (1–34)-induced suppression of COL10A1 expression (Fig. 5), indicating that PKC activity is not or only to a low extent involved in this effect.

PTH (1–34) Induces c-fos Expression in Hypertrophic Chondrocytes

Freshly-isolated hypertrophic and proliferating chondrocytes were incubated with PTH (1–34) at 10^{-8} M. RNA was collected after 30, 60, and 180 min and analyzed by Northern hybridization using a probe for human c-fos. C-fos expression was detected 30 min after adding PTH (1–34) and was maintained for at least 3 h (Figs. 6 and 7). A similar result was obtained with PTHrP (1–40) (Fig. 7), but not with other PTH fragments. Only low levels of c-fos were induced in a fraction of proliferating chondrocytes derived from fetal bovine epiphyses (not shown).

No significant levels of c-fos were induced when hypertrophic chondrocytes were incubated with PTH (3–34), confirming the notion that PKA was predominantly responsible for c-fos induction. The induction of c-fos by PTHrP suggested a role of c-fos in suppression of collagen X expression. In order to verify this assumption, hypertrophic chondrocytes were transfected with a RSV-c-fos expression vector and incubated for 24 and 48 h under serum-free conditions. Northern hybridization analysis, however, showed only a low, but reproducible reduction of α1(X) mRNA levels in c-fos transfected cells in comparison to control cells (not shown) due to low transfection efficiency. Control of transfection rates of fetal bovine chondrocytes with a RSV-GFP expression vector revealed that the transfection rates did not exceed 5–8% of the cells when using different transfection agents, including lipofectin and fugene. Therefore, the role of c-fos in regulating collagen X expression was investigated using reporter genes driven by the COL10A1 promoter (see below).

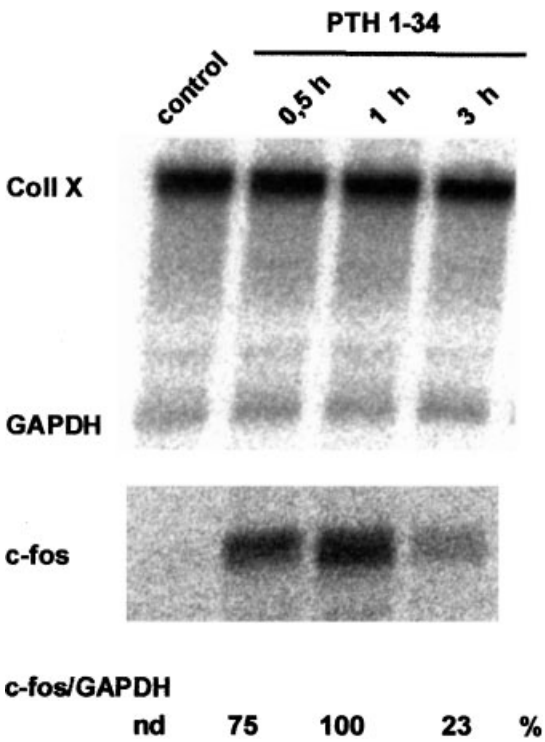

Fig. 6. Induction of c-fos by PTH (1–34) in bovine chondrocytes. Chondrocytes derived from the hypertrophic zone and the proliferating zone chondrocytes were incubated with PTH (1–34) for 30–180 min, total RNA was extracted and analyzed by Northern hybridization for c-fos, α1(X) collagen and GAPDH expression; c-fos mRNA was detected 30 min after addition of PTH (1–34), while collagen X mRNA started declining during the first 3 h (see also Fig. 3).

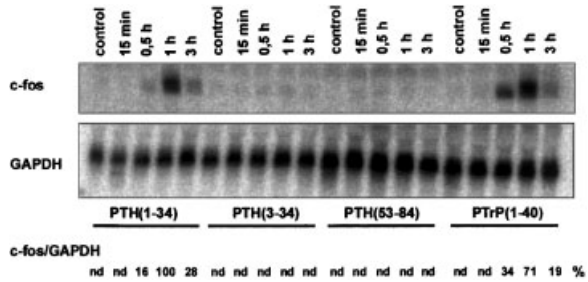

Fig. 7. c-fos is induced by PTH (1–34) and PTHrP (1–40), but not by PTH (3–34). Chondrocytes were incubated with PTH fragments as in Figure 6, and onset of c-fos expression was monitored by Northern hybridization. PTH (3–34) did not induce c-fos to a significant extent, indicating that c-fos induction in these cells is mediated by the adenylate cyclase-PKA pathway.

Characterization of a 530-bp Enhancer Element in the Human Col10a1 Gene

In previous studies on the transcriptional regulation of the human type X collagen gene, a tissue-specific, strong enhancer region has been located in the Col10a promoter between − 873- and − 2,410-bp upstream of the transcription start site [Thomas et al., 1995; Chambers et al., 1996; Beier et al., 1997]. Luciferase reporter gene constructs bearing this region revealed high transcription rates after transient transfection into growth plate chondrocytes, but not in chondrocytes from the resting zone or other cell types [Beier et al., 1997]. Furthermore, a tissue-specific silencer element was postulated between − 2,400- and − 2,986-bp upstream of the transcription start site, which reduced

transcription of the reporter gene in non-hypertrophic cells and fibroblasts, but was inactive in hypertrophic chondrocytes [Beier et al., 1997].

In order to show position-independent activity of this enhancer, human collagen X promoter fragments located between – 868 and – 2407 or – 2861 bp, respectively, were ligated to the 3′ end of the luciferase gene (pGL2500inv and pGL3000inv, Fig. 1). Furthermore, a 537-bp fragment (– 1,870 to – 2,407 bp) was generated by PCR and combined with the 5′ end of pGL900 (PGL900-1/8) or in reverse direction downstream of the reporter gene pGL900 (pGL900-530) (see Fig. 1). These reporter gene constructs were transiently transfected into hypertrophic chondrocytes together with β-gal control constructs for internal standardization, and luciferase activity in the cell extracts was measured after 48 h.

The reporter constructs pGL2500 and pGL3000 were about 100-fold more actively transcribed in bovine growth plate chondrocytes than the reporter genes, pGL500 and pGL900, containing only the proximal promoter region up to – 900 bp upstream of the transcription start site (Fig. 8A), confirming the presence of an unusually strong, tissue-specific enhancer [Beier et al., 1997]. The activity of the enhancer was independent from ist position as it was retained in pGL2500inv and pGL3000inv (Fig. 8A). More than 50% of the activity of pGL2500 was retained in the construct pGL900-1/8 (Fig. 8B), indicating that the major enhancer activity is located between – 1,870 and – 2,407 bp; the same transcriptional activity was obtained with pGL900-530, confirming that this enhancer activity was also independent from position and direction (Fig. 8A).

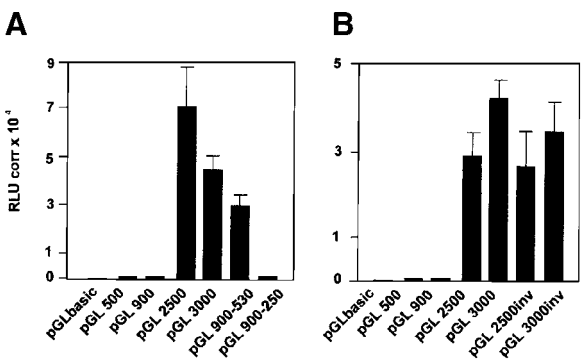

Fig. 8. Identification of a strong enhancer element in the human COL10A1 gene. Bovine growth plate chondrocytes were transiently transfected with luciferase reporter gene constructs containing various fragments of the human COL10A1 gene as shown in Figure 1, and cotransfected with the pSVβgal vector to standardize transfection efficiency. Luciferase activity was measured after 24 h and normalized to β-gal values. Transcriptional activity of cells transfected with pGL2500 and pGL3000 was about 50 to 100 times higher than those transfected with promoter constructs lacking the 530-bp enhancer element. (**A**) Transcriptional activity was retained by at least 50% in the pGL900-530 construct containing the 530-bp element downstream of the luciferase reporter. (**B**) Transcriptional activity was unaltered, when the long promoter fragments were positioned downstream of the luciferase gene (pGL2500 inv and pGL3000inv).

PTH (1–34) and PTHrP (1–40) Inhibit Type X Collagen Expression at Transcriptional Level

For identification of PTH/PTHrP-responsive elements in the human COL10A1 gene, the above reporter gene constructs were transiently transfected into hypertrophic chondrocytes, and the cells incubated for 24 h in the presence and absence of PTH (1–34) and PTHrP (1–40). PTH (1–34) at 10^{-8} M as well as PTHrP (1–40) substantially inhibited transcription of pGL3000, pGL2500, and pGL900-1/8. Transcription of shorter promoter fragments pGL500 and pGL900 was not inhibited; in contrast, a slight stimulation of pGL 900 was reproducibly observed in the presence of PTH (1–34) (Fig. 9). Also, transcription of pGL3000 containing the

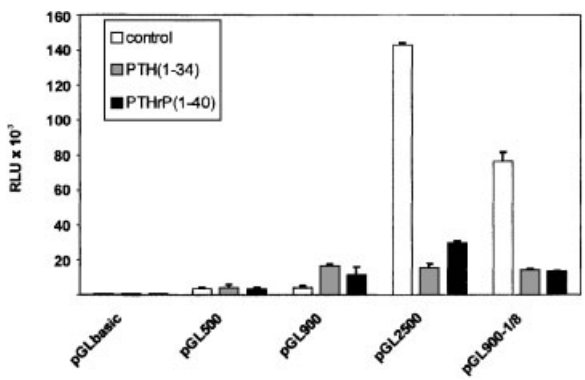

Fig. 9. PTH (1–34) and PTHrP (1–40) suppress transcription of human COL10A1 promoter-luciferase constructs. Bovine growth plate chondrocytes were transfected with the promoterless pGLbasic or with the human COL10A1 reporter constructs pGL2500, pGL900, pGL 900-1/8, and pGL500 for 6 h, and incubated with or without PTH (1–34) or PTHrP (1–40) at 10^{-8} M for further 48 h; β-galactosidase gene activity was measured and used for normalizing the transfection rates. Transcriptional activity of the promoter constructs was measured by the luciferase activity of the cell extracts. The bars represent mean values of triplicate cell culture dishes. PTH (1–34) and PTHrP (1–40) both caused a downregulation of the transcriptional activity of the COL10A1 promoter constructs pGL2500 and pGL900-1/8.

silencer element [Beier et al., 1997] was inhibited by PTH (1–34) (data not shown); since, the same inhibitory effect was seen with pGL2500 construct, the suppressive effect of PTH (1–34) does not seem to involve the silencer element located between −2,407 and −2,864 bp. The data rather indicate the presence of the PTH (1–34) responsive element in the 530-bp enhancer.

Overexpression of c-fos Suppresses Transcription of Col10A1 Enhancer-Containing Reporter Gene Constructs

The same pattern of transcription inhibition of the enhancer-containing reporter constructs pGL2500 and pGL900-1/8 was achieved when hypertrophic chondrocytes were transiently cotransfected with a c-fos expression vector and the reporter gene constructs (Fig. 10). The transcription rate of reporter gene constructs lacking the 530-bp enhancer, such as the pGL900 were not reduced by overexpression of c-fos, supporting the notion that the 530-bp enhancer element is involved in c-fos-mediated inhibition effect of collagen X gene transcription by PTH/PTHrP. C-fos may act through one or several of the seven putative AP-1 sites predicted by computer search of the enhancer sequence.

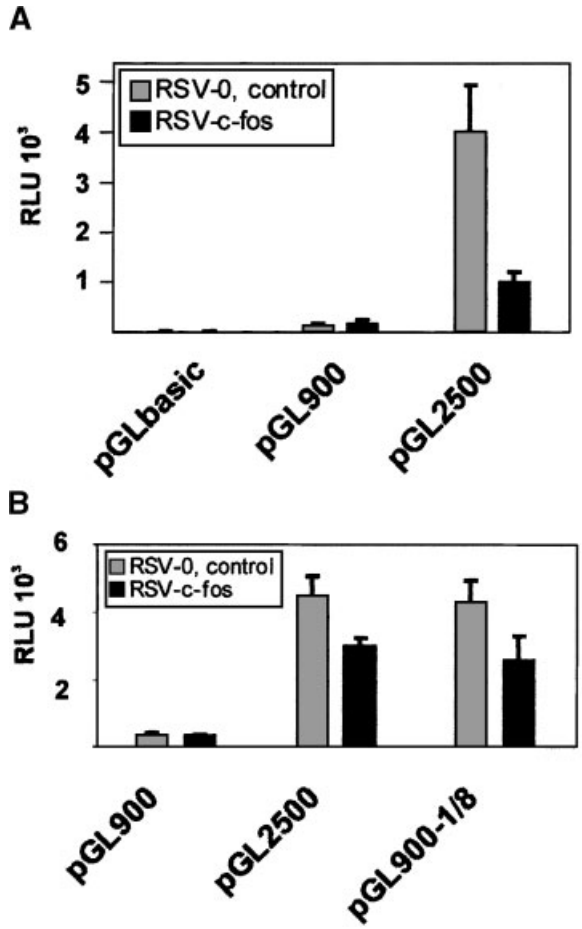

Fig. 10. Overexpression of c-fos suppresses transcriptional activity of the COL10A1 enhancer. Bovine growth plate chondrocytes were transfected with 10 μg of either pRSV-cfos expression vector or the pRSV control vector and cotransfected with pSVβgal and the luciferase reporter gene pGL900, pGL900-1/8, and pGL2500. Luciferase activity was measured after 48 h and normalized to β-gal activity. The data show that c-fos overexpression suppresses transcription of the same reporter genes, pGL 900-1/8 and pGL2500, as incubation of chondrocytes with PTH or PTHrP.

DISCUSSION

Our data indicate a central role of c-fos in the negative regulation of type X collagen expression by PTH and PTHrP. Both hormones induce c-fos in hypertrophic chicken and bovine chondrocytes [Ionescu et al., 2001 and this study], and both PTH and PTHrP suppress α1(X) mRNA steady state levels in fetal bovine growth plate chondrocytes within few hours, with a maximal suppression after 48 h, while onset of c-fos was seen already 30 min after addition of PTH (1–34). Furthermore, PTH and PTHrP inhibited transcription of COL10A1 reporter genes containing a tissue-specific enhancer element after transient transfection into hypertrophic chondrocytes. Transcription of the same reporter genes, but not of reporter genes that did not contain the enhancer element was inhibited by overexpression of c-fos. This tissue-specific enhancer element, which was further narrowed down to a 530-bp region between −1,870- and −2,407-bp upstream of the transcription start site contains seven putative AP−1 sites; one or several of them may be involved in the c-fos-mediated inhibition of collagen X expression.

The inhibitory effect was mediated by the aminoterminal fragments, PTH (1–34) and PTHrP (1–40), respectively, while PTH (3–34) was not active, supporting the notion that the adenylate cyclase/PKA pathway of the PTH/PTHrP receptor is largely responsible for the suppression of COL10A1 expression. This finding is consistent with the short-term stimulation of cAMP activity and alkaline phosphatase activation in rabbit or chick growth plate chondrocytes by PTH (1–34), but not by PTH (3–34) [Iwamoto et al., 1994; Tsukazaki et al.,

1996; Schwartz et al., 1997]. It cannot be excluded, however, that the PKC/PLC-pathway of the PTH/PTHrP receptor, which is activated by the midregional domain PTH (28–34) [Jouishomme et al., 1992, 1994] is also partially involved, although blocking the PKC activity with *bis*-indolylmaleimide did not affect the suppressive effect of PTH (1–34). Recent results by Takasu et al. [1999], challenged the concept of an exclusive activation of the PLC/PKC signalling pathway by the midregional domain, 28–34, by showing that recombinant human PTH (1–31) was also able to stimulate phospholipase C in porcine LLC-PK1 cells.

In order to identify PTH/PTHrP-responsive elements in the human type X collagen gene, various luciferase reporter gene constructs were prepared with fragments derived from the human COL10A1 promoter and transfected into hypertrophic chondrocytes. A tissue-specific enhancer fragment, which we had characterized previously [Beier et al., 1997] was further narrowed down to a 530-bp fragment located between − 1,870- and − 2,407-bp upstream of the transcription start site. Reporter gene constructs containing this 530-bp enhancer element showed high transcription activity in hypertrophic, but not in resting chondrocytes. The activity was also retained in constructs containing the enhancer at the 3′ end of the reporter gene or in reverse direction, supporting the notion that the enhancer activity is position- and direction-independent.

Interestingly, transcription of all reporter gene constructs bearing this enhancer (pGL2500, pGL3000, and pGL900/1–8), but not of promoter fragments lacking the enhancer, was significantly inhibited when the transiently transfected hypertrophic chondrocytes were treated with PTH (1–34) or PTHrP (1–40), indicating the presence of a PTH/PTHrP-responsive element in the enhancer.

In transient transfection experiments, transcription of the shorter reporter gene constructs, pGL500 and pGL900, was upregulated to some extent by PTH (1–34) compared to control cultures. Studies on the chick and human type X collagen promoter have shown the existence of additional enhancing elements in the 5′ proximal region of the promoter [Luvalle et al., 1993; Dourado and Luvalle, 1998; Volk et al., 1998; Beier et al., 1999] and in the first intron [Beier et al., 1997], together with additional silencer elements [Long and Linsenmayer, 1995; Long et al., 1998]. Although, the promoter structure of chicken, human, bovine, and murine Col10a1 genes differ considerably [Beier et al., 1996], these proximal enhancer elements sequences or additional, as yet unidentified elements could confer the stimulatory effects of PTH (1–34) on transcription of the pGL900 element through either of the two signalling pathways activated by the PTH/PTHrP receptor. This is in line with the presence of numerous putative AP–1 and AP–2 binding sites in the murine and chicken Col10a1 promoter [Beier et al., 1996], and other sites known to be regulated by the PTH/PTHrP receptor-induced signals. Harada et al. [1997] have shown that an AP1-binding element in the Col10a1 promoter activates type X collagen transcription in a rat calvarial cell line. AP-1 binding also plays a role in the transcriptional activation of some matrix metalloprotease genes, such as MMP9 by PTH [Kawashima-Ohya et al., 1998; McClelland et al., 1998].

Although, the role of PTH/PTHrP in inhibition of type X collagen synthesis and chondrocyte differentiation to hypertrophic cells has been known for some time, the mechanism and signalling pathways involved are still obscure. Recently, PTHrP has been shown to induce CREB phosphorylation and c-fos protein production in chick sternal chondrocytes [Ionescu et al., 2001]. This is in line with the activation of protein kinase A (PKA) through the adenylate cyclase pathway by the aminoterminal fragments of PTH/PTHrP. PKA has been shown to phosphorylate CREB, which may stimulate transcription c-fos gene by binding to its CRE element [Pearman et al., 1996; McCauley et al., 1997; Tyson et al., 1999]. The fact that transcription of the same reporter gene constructs pGL2500 and pGL 900-1/8 which respond to PTH(1–34) and PTHrP(1–40) was also inhibited by overexpression of c-fos strongly suggests that the effect of PTH/PTHrP on type X collagen expression is mediated by c-fos.

Transcription of most AP–1-regulated genes is upregulated by c-fos/c-jun heterodimers. In fact, a regulatory AP–1-site involved in Osteogenic Protein-1 (OP-1)-induced stimulation of type X collagen gene expression has been identified in the proximal promoter of the murine Col10a1 gene [Harada et al., 1997]. In our study, however, we show that transcription of type X collagen reporter genes in hypertrophic chondrocytes as well as type X collagen mRNA levels are inhibited by overexpression of

c-fos. The question remains whether transcriptional regulation of type X collagen genes occurs by direct binding of AP1 factors to COL10A1 DNA, or indirectly through c-fos induced genes. Direct regulation of type X collagen gene transcription by AP-1 factors, however, cannot be excluded.

A homology search for putative transcription factor binding sites revealed seven putative AP1-binding sites in the enhancer, which may be recognized by c-Fos. Whether one or several of these AP 1 sites is involved in transcriptional regulation of COL10A1 and bind c-fos or other AP-1 factors is currently under investigation.

The results presented here as well as by other investigators recently indicate a rather complex regulation of type X collagen gene expression. Several extracellular signalling molecules, such as PTH/PTHrP, T3 [Bohme et al., 1992; Ballock and Reddi, 1994], BMPs [Grimsrud et al., 1998, 2001] and the transcription factor Cbfa1 [Kim et al., 1999; Enomoto et al., 2000] appear to control type X collagen transcription, as do multiple intracellular signalling pathways including several MAP kinases, PKA, and PI3 kinase [Beier and LuValle, 1999; Beier et al., 1999a,b]. Complete elucidation of the pathways, transcription factors, and promoter elements involved in these effects will be required to obtain a better understanding of the mechanisms regulating type X collagen expression and chondrocyte differentiation.

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