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Regulatory Mechanism of Human Connective Tissue Growth Factor (CTGF/Hcs24) Gene Expression in a Human Chondrocytic Cell Line, HCS-2/8[1]

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CTGF/Hcs24 is a multi-functional growth factor that potentiates either the growth or differentiation of mesenchymal cells, according to the biological conditions. Among various functional aspects of CTGF/Hcs24, it is especially notable that CTGF/Hcs24 may promote endochondral ossification in growth cartilage through all stages, and it is highly expressed in a human chondrosarcoma-derived chondrocytic cell line (HCS-2/8). In this study, to clarify the regulatory mechanism of CTGF/Hcs24 gene expression in chondrocytes, we analyzed the transcriptional activity of the CTGF/Hcs24 promoter and the effect of the CTGF/Hcs24 3′-untranslated region (3′-UTR) on gene expression in HCS-2/8 by means of an established DNA transfection and luciferase reporter gene assay system. As a result, the luciferase activity of the CTGF/Hcs24 promoter was found to be remarkably high in HCS-2/8. The 3′-UTR of the CTGF/Hcs24 gene strongly repressed the luciferase activity in HCS-2/8, when it was linked to the downstream of the luciferase reporter gene, suggesting its functionality also in chondrocytic cells. Deletion analysis of the CTGF/Hcs24 promoter clarified a major segment responsible for the enhanced CTGF/Hcs24 promoter activity in HCS-2/8. The TGF-β response element in the DNA segment was active in HCS-2/8, and point mutations in the element moderately decreased the highly maintained promoter activity with total loss of TGF-β responsiveness. These results indicate that the strong expression of the CTGF/Hcs24 gene in HCS-2/8 was mainly caused by high transcriptional activity of the CTGF/Hcs24 promoter, and that the TGF-β response element is one of the critical elements that support the high transcription activity.

Key words: CCN family, chondrocyte, CTGF, HCS-2/8, transcription.

Connective tissue growth factor/ hypertrophic chondrocyte specific gene product 24 (CTGF/Hcs24), a member of the CCN (*i.e.*, CTGF/CYR61/NOV) family, is a multiple functional growth factor, which promotes cell proliferation, chemotaxis, adhesion and extracellular matrix (ECM) formation of mesenchymal cells (*1–6*). These CCN proteins are involved in both physiological and pathological processes, such as chondrogenesis (*4, 7–11*), angiogenesis (*12–15*), embryogenesis (*16*), wound healing (*17*), tumorigenesis (*11, 15, 18*), and fibrotic disorder like atherosclerosis (*19*) and systemic sclerosis (*20*).

CTGF/Hcs24 mRNA or protein is expressed in diverse cell types, such as chondrocytes (*7, 9–11, 21*), fibroblasts (*1, 17*), (vascular) endothelial cells (*12–14, 22*), vascular smooth muscle cells (*12–14, 22*), epithelial cells (*23*), tumor cells (*18, 24, 25*), and the central nervous system (*26–28*) in certain conditions. Of note is that the highest expression is observed in hypertrophic chondrocytes (*4, 7, 29*). Among the multiple properties of CTGF/Hcs24, its function in endochondral ossification is especially worthy of note, because CTGF/Hcs24 is a unique growth factor that promotes the proliferation and differentiation of chondrocytes at many stages (*4, 7, 8, 12, 13, 29*). An mRNA predominantly expressed in HCS-2/8 was cloned by differential display polymerase chain reaction (PCR), and named *hcs24* (*29*). The *hcs24* transcript was then found to be identical to a CTGF/Hcs24 gene transcript. CTGF/Hcs24 is highly expressed in rabbit growth cartilage and HCS-2/8 cells in culture, which was shown by Northern blot analysis. As stated above, the highest level of CTGF/Hcs24 expression was observed at the hypertrophic stage in chondrocytes, whereas relatively lower levels were found at earlier stages, as shown by *in situ* hybridization analysis of growth cartilage and reverse

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Abbreviations: CTGF, connective tissue growth factor; Hcs24, hypertrophic chondrocyte specific gene product 24; ECM, extracellular matrix; HCS-2/8, human chondrosarcoma cell line clone-2/8; TGF-β, transforming growth factor-β; 3′-UTR, 3′-untranslated region

transcriptase-mediated PCR (RT-PCR) analysis of rabbit primary cell cultures (*29*). A recombinant human CTGF/Hcs24 protein promoted not only the proliferation of rabbit growth cartilage and HCS-2/8 cells, but also mRNA expression of aggrecan core protein, and type II and type X collagens in both types of cells (*7*). The exogenous recombinant CTGF/Hcs24 also increased the alkaline phosphatase activity in rabbit growth cartilage cells. It was also shown that CTGF/Hcs24 promotes angiogenesis, which is required at the final stage of endochondral bone formation (*12, 13*). Moreover, CTGF/Hcs24 also stimulates the proliferation and differentiation of osteoblasts, which are involved in the final stage of endochondral ossification (*8*). Therefore, CTGF/Hcs24 may be called "ecogenin," an endochondral ossification genetic factor.

The regulatory mechanism of differential expression of CTGF/Hcs24 in chondrocytes remains unclear. Analysis of the human CTGF/Hcs24 gene promoter in fibroblasts revealed a novel transforming growth factor-β (TGF-β) response element (*30, 31*), although the protein factor(s) that interacts with this DNA segment has not yet been identified. In chondrocytes and osteoblasts, CTGF/Hcs24 is induced not only by TGF-β but also by bone morphogenetic protein-2, a member of the TGF-β family (*8, 29*). In addition to the promoter-coupled element, a *cis*-acting element of structure-anchored repression (CAESAR) in the 3′-untranslated region (3′-UTR) of the CTGF/Hcs24 gene was discovered, which confers post-transcriptional repression (*32–34*), suggesting the complexity of CTGF/Hcs24 gene regulation. Of importance is that CTGF/Hcs24 is highly expressed in HCS-2/8 cells, which retain characteristics of chondrocytes in culture, and thus they may be useful for dissecting the regulation mechanism of the differential expression of CTGF/Hcs24 in chondrocytes (*35–38*). In the present study, to clarify the controlled expression mechanism of CTGF/Hcs24 in chondrocytes, we functionally analyzed the two major cis-acting regulators of the CTGF/Hcs24 gene expression—*i.e.* the CTGF/Hcs24 promoter and the CTGF/Hcs24 3′-UTR, in chondrocytic HCS-2/8 cells.

MATERIALS AND METHODS

Cell Culture—HCS-2/8 and HeLa cells were used. HCS-2/8 stands for human chondrosarcoma-2/8, a clonal cell line established in 1989 by M. Takigawa *et al.* (*35–38*). It shows characteristics of immature to pre-hypertrophic chondrocytes in cell culture. HeLa is a fibroblastic cell line derived from a human cervical cancer. HCS-2/8 and HeLa were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under humidified air containing 5% CO_2.

Labeling Cells with L-[^{35}S]Methionine—Half a million HCS-2/8 cells were seeded into a 3.5 cm tissue culture dish and then cultured to confluency. After being starved in methionine-free DMEM supplemented with 5% dialyzed FBS for 16 h, the cells were labeled with 200 μCi (7.4 MBq)/ml of L-[^{35}S]methionine for 2 h at 37°C. The cells were then washed with Dulbecco's phosphate-buffered saline (PBS), lysed in a RIPA buffer (*39, 40*), scraped, and collected from the cell culture dish.

Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—Anti-CTGF/Hcs24 or pre-immune rabbit serum was added to the cell lysate in the RIPA buffer. Protein-G-Sepharose 4B (Amersham/Pharmacia Biotech, Buckinghamshire, UK) was added and suspended, and the suspension was swirled at 4°C for 3 h. The immunocomplex was washed six times with three different buffers (*39, 40*), and SDS sample buffer with 2.5% 2-mercaptoethanol was added to dissociate proteins as described previously (*39*). After boiling at 95°C for 5 min, the immunoprecipitated proteins were separated by SDS-PAGE with 15–25% MULTIGEL (Daiichi Pure Chemicals, Tokyo) and then fluorographed, using a commercially available reagent (Amplify, Amersham/Pharmacia Biotech).

RNA Extraction and Reverse Transcriptase-Mediated PCR (RT-PCR)—Total RNA was extracted from growing HCS-2/8 and HeLa cells at the logarithmic phases by an acid guanidium phenol-chloroform (AGPC) method (*41*). Two primers specific for the 3′-UTR portion of CTGF/Hcs24 were used for RT-PCR, as described previously (*32*). The sense primer recognizes the very end of the CTGF/Hcs24-coding frame. The antisense primer recognizes immediately upstream of the putative poly(A)$^+$ additional signal. Reverse transcription with avian myelosarcoma virus (AMV) reverse transcriptase was carried out using a commercially available kit (Takara Shuzo, Tokyo) with 1.5 μg of total RNA and the antisense primer. Subsequent PCR amplification was performed with the same experimental kit and primers, following the manufacturer's protocol. Each amplification cycle consisted of 1 min at 94°C, 30 s at 50°C, and 1 min at 72°C. After 30 cycles of a chain reaction and subsequent incubation at 72°C for 5 min, the PCR products were analyzed by 1.5% agarose gel electrophoresis.

Enzyme-Linked Immunosorbent Assays (ELISA)—Cells were inoculated at a density of 1×10^6 into a 10-cm-diameter dish and then cultured in DMEM containing 10% FBS for 36 h. After the medium had been changed to 2 ml of DMEM containing 10% FBS, they were cultured for 12 h continuously until the sampling time-point, and a cell lysate was evaluated with a CTGF/Hcs24 ELISA system (*42*). In this system, highly specific monoclonal antibodies against CTGF/Hcs24 had been raised and utilized. The cell lysate was prepared in another RIPA buffer (50 mM Tris-Cl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) containing aprotinin, leupeptin and PMSF as protease inhibitors. CTGF/Hcs24 present in proliferative cells was represented as the relative amount (ng) of CTGF/Hcs24 in 1×10^6 cells.

Molecular Clones—In pTS589 (provided by Japan Tobacco) (Fig. 3A), a human CTGF/Hcs24 promoter was connected to a firefly luciferase reporter gene for evaluation of its activity in a variety of cells. All the deletion mutants were derived from pTS589. CTGF/Hcs24 promoter subfragments were amplified by PCR from pTS589, using combinations of five different sense primers and an anti-sense primer. The nucleotide sequences of the sense primers were as follows:

CTPDS1: 5′-GGGGTACCTGCTGTTTGCCTCTTCAG-3′,
CTPDS2: 5′-GGGGTACCATGAATCAGGAGTGGTGCGA-3′,
CTPDS3: 5′-GGGGTACCTGTGAGCTGGAGTGTGCCAG-3′,
CTPDS4: 5′-GGGGTACCGTCCCTGTTTGTGTAGGACT-3′,

CTPDS5: 5′-GGGGTACCGGAGCGTATAAAAGCCTCG-3′.

Each sense primer comprises a flanking *Kpn*I site for the subsequent subcloning process. The anti-sense primer, LUCA (5′-CCAGCGGTTCCATCTTCCAG-3′), recognizes a firefly luciferase gene sequence immediately downstream of the initiation codon. The resultant amplicon with one of the CTPDS sense primers and the LUCA anti-sense primer is composed of a deleted CTGF/Hcs24 promoter fragment with a small portion of the luciferase gene, which juxtapose at the *Hin*dIII junction site. Each promoter fragment was digested with *Kpn*I and *Hin*dIII, and then subcloned between the corresponding sites in pTS589, replacing the original promoter fragment. The resultant plasmids were termed pDS1-5. The structures of these plasmids are illustrated in Fig. 5A.

A pTS589 mutant with 4-point mutations in the TGF-β response element of the CTGF/Hcs24 promoter was also constructed. A brief schema of the construction method is presented in Fig. 6A. Two primers, TbREmtF and TbREmtR, were designed and synthesized to construct the mutant. The nucleotide sequences of these primers were as follows:

TbREmtF: 5′-**TTCTTAAGGTGTCAGGATCAATCCGGT**-3′

TbREmtR: 5′-**ACCTTAAGAATCAGCATTCCTCCGTCT**-G-3′

Bold: mutated nucleotide sequences for point mutations, underlined: an *Afl*II site inscribed for construction

Two divided promoter fragments were amplified with the following primer pairs, SEQGL/TbREmtR (the upstream fragment) and TbREmtF/LUCA (the downstream fragment) from pTS589. The additional primers used here were:

SEQGL: 5′-TGTCCCCAGTGCAAGTGCAG-3′
LUCA: described in the last paragraph.

Both of these PCR products were digested with *Afl*II (New England Biolabs, Beverley, MA, USA) and then ligated together with DNA ligation kit ver. 2 (Takara Biomedicals). The ligated DNA fragment was amplified by PCR with SEQGL and LUCA (the outside pair), digested with *Kpn*I and *Hin*dIII, and then subcloned between the corresponding sites in pTS589, replacing the original promoter fragment. The resultant plasmid was termed pTS589mtTbRE.

The plasmids, pGL3UTRS and pGL3UTRA (Fig. 4A), were constructed by inserting the CTGF/Hcs24 3′-UTR at the end of a luciferase gene driven by an SV40 promoter in the backbone of pGL3-control (Promega, Madison, WI, USA), as described previously (*32*). In pGL3ΔP, the SV40 promoter region had been removed from pGL3-control. Internal control vectors, pRL-CMV and pRL-TK were also commercially available (Promega). These plasmids contain *Renilla* luciferase genes under the control of the cytomegalovirus (CMV) enhancer/promoter and the herpes simplex virus thymidine kinase (HSV-TK) promoter, respectively.

DNA Transfection and Luciferase Assay—HCS-2/8 and HeLa cells were transfected with a cationic liposome reagent, *i.e.* Fugene™ 6 transfection reagent (Roche, Basel, Switzerland) for HCS-2/8 and LipofectAMINE (Lifetechnologies, Rockville, MD, USA) for HeLa. The amount of transfected plasmids, and the pre-transfection period after seeding and the post-transfection period before harvesing were optimized for HCS-2/8 for the first time in this study. For HeLa, we utilized the manufacturer's optimized protocol. The total amount of transfected plasmid was standardized as 2 μg for each experiment in a 35 mm diameter tissue culture well with log-phase growing cells. In a few experiments, DNA transfection was carried out on a half scale.

A Dual luciferase assay system (Promega) was applied for sequential measurement of firefly and *Renilla* luciferase activities with specific substrates, *i.e.* beetle luciferin and coelenterazine, respectively. Quantification of luciferase activities and calculation of relative ratios were carried out manually with a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA).

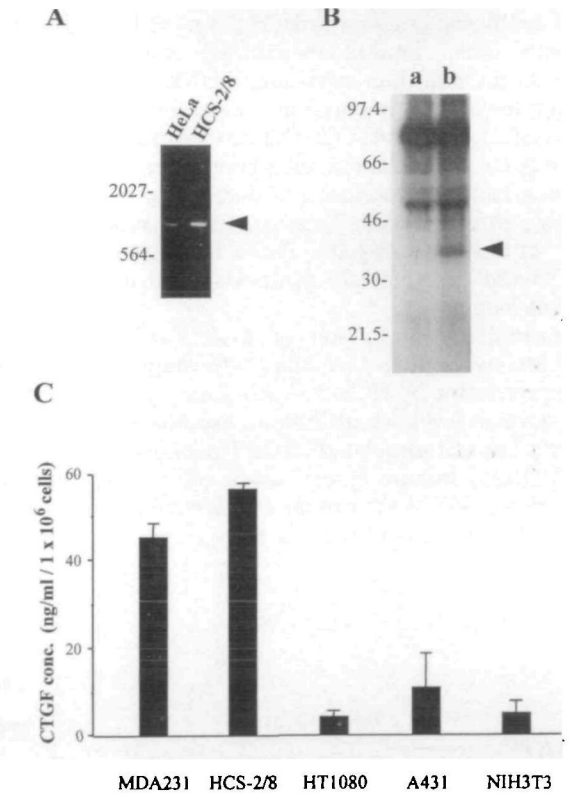

Fig. 1. **Detection of CTGF/Hcs24 expression in HCS-2/8 cells.** (A) Agarose gel electrophoresis analysis of the 3′-UTR portion of CTGF/Hcs24 cDNA amplified by RT-PCR. Total RNA was extracted from log-phase growing HeLa cells and HCS-2/8 cells. RT-PCR was carried out with primers specific for the CTGF/Hcs24 3′-UTR. Two microliters out of a total reaction mixture of 100 μl was analyzed. The arrowhead at the right indicates the position of the major amplicon. (B) A protein with an apparent molecular weight of 36–38 kDa specific for anti-CTGF/Hcs24 antiserum. Proteins were labeled with L-[^{35}S]methionine, immunoprecipitated with anti-CTGF/Hcs24 serum, separated by SDS-PAGE, and then fluorographed (lane b). Preimmune serum was used as a control (lane a). The arrowhead indicates the major band that only appeared in lane b. (C) Cell-associated CTGF/Hcs24 in various cell lines measured with the ELISA system. Cells were cultured in DMEM containing 10% FBS as described under "MATERIALS AND METHODS." Cell lysates were prepared in a RIPA buffer. The concentration of CTGF/Hcs24 associated with proliferative cells was calculated as the relative amount (ng) per 1 × 10^6 cells.

RESULTS

Detection of CTGF/Hcs24 mRNA in HCS-2/8 Cells—Using primers that were designed to recognize the 3′-UTR region of human CTGF/Hcs24 mRNA, RT-PCR was performed to confirm the expression of the CTGF/Hcs24 gene. As shown in Fig. 1A, single major bands corresponding to approximately 1.0 kbp were detected on agarose gel electrophoresis of the crude RT-PCR products derived from RNA of both HCS-2/8 and HeLa cells. Also the CTGF/Hcs24 mRNA expression level in HCS-2/8 was found to be higher than that in HeLa cells. Direct sequencing analysis of these single band DNAs revealed their identity as the human CTGF/Hcs24 3′-UTR (data not shown).

Detection of CTGF/Hcs24 at the Protein Level with CTGF/Hcs24 Antiserum—Although the mRNA expression was confirmed, there has been little protein-level evidence for the CTGF/Hcs24 in HCS-2/8 cells. To ascertain CTGF/Hcs24 synthesis, proteins in HCS-2/8 were labeled with L-[^{35}S]methionine, immunoprecipitated with anti-CTGF/Hcs24 serum, and then separated by SDS-PAGE (Fig. 1B). Pre-immune serum was used as a control (lane a). The protein specific for the anti-CTGF/Hcs24 serum only appeared in lane b, *i.e.* not in lane a, as a band corresponding to an apparent molecular weight of 36–38 kDa, which is indicated by an arrowhead. This experiment was repeated 5 times, with similar results. These findings indicate that CTGF/Hcs24 was actively synthesized during a labeling period as long as 2 h.

Quantitative Comparison of Cell Associated CTGF/Hcs24 among Various Cell Lines—To compare the CTGF/Hcs24 production by HCS-2/8 with those by other cell lines at the protein level, we utilized an established ELISA system (*42*). The cell associated CTGF/Hcs24 levels in HCS-2/8 cells, MDA231 human breast cancer cells, HT1080 human fibrosarcoma cells, A431 human epidermoid carcinoma cells and NIH3T3 murine fibroblasts were compared. As shown in Fig. 1C, the highest level of CTGF/Hcs24 was observed in HCS-2/8 cells, it being more than 13-fold, 5-fold, or 11-fold higher than that in HT1080, A431, or NIH3T3 cells, respectively. It was even higher than in MDA231 cells, which were reported previously to show a high level of CTGF/Hcs24 production, as confirmed here as well (*24*).

Establishment of an Efficient DNA Transfection System for HCS-2/8 Cells—For DNA transfection experiments, we established an efficient DNA transfection system for HCS-2/8. Firstly, we confirmed a sufficient level of luciferase expression in HCS-2/8 by transient transfection with 2 μg of pGL3-control (Fig. 2, A and B, and unpublished data). Secondly, the pre-transfection period after cell seeding was optimized. HCS-2/8 cells were cultured for 24 h or 48 h before being transfected with 2 μg of pGL3-control, and then harvested 48 h after transfection. Then, firefly luciferase activities were measured. The cells with a 24 h pre-transfection period showed a higher response to DNA transfection, as measured as luciferase activities, compared to 48 h (Fig. 2C). Therefore, a 24 h pre-transfection period after seeding was chosen for the following transfection experiment. Finally, the effect of the post-transfection period before harvesting was optimized. Firefly luciferase activities of cells with post-transfection periods of 24 h, 48 h, and 72 h were compared. Luciferase activity showed the highest value at 48 h post-transfection among these three periods (Fig. 2D). Based on these results, all the following transfection experiments with HCS-2/8 cells were performed with a 24 h pre-transfection period after seeding and a 48 h post-transfection period before harvesting.

CTGF/Hcs24 Promoter Activities in HCS-2/8 Compared to HeLa—To evaluate the transcription activity of the CTGF/Hcs24 promoter in HCS-2/8 cells, HCS-2/8 or HeLa cells were co-transfected with 1.0 μg of pTS589 and 1.0 μg of pRL-TK (internal control), or 1.5 μg of pTS589 and 0.5 μg of pRL-CMV (internal control) per 35 mm diameter tissue culture well. Firefly luciferase and *Renilla* luciferase activities were measured, and relative luminescence values

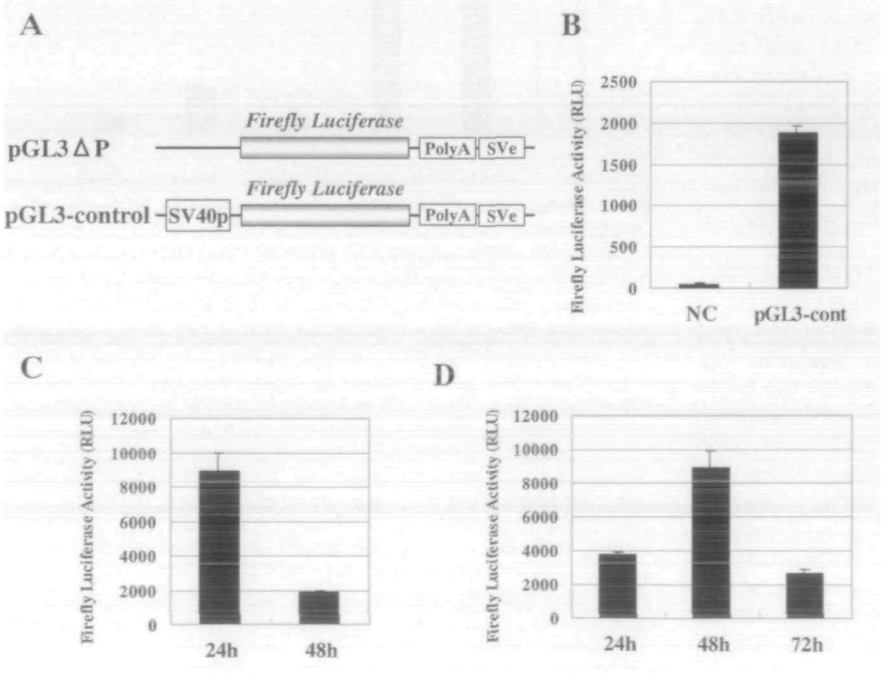

Fig. 2. **(A) Schemas of the plasmids used for optimization of the transient expression conditions in HCS-2/8 cells.** SV40p, poly A, and SVe denote the SV40 promoter, polyadenylation signal, and enhancer, respectively. **(B) Expression of firefly luciferase from the plasmids illustrated in panel A in HCS-2/8 cells.** HCS-2/8 cells were transfected 48 h after being seeded and harvested 48 h after transfection. NC: negative control with pGL3ΔP. **(C) Effect of the pre-transfection period after seeding.** Two time points (24 h and 48 h) were comparatively examined. This experiment was performed with a fixed 48 h post-transfection period before harvesting. **(D) Effect of the post-transfection period before harvesting.** Three time points (24 h, 48 h, and 72 h) were comparatively examined. In this experiment, the pre-transfection period after seeding was fixed at 24 h. All the values are represented as relative luminescent units (RLU), as measured with a luminometer.

for firefly luciferase *versus Renilla* luciferase were calculated with a luminometer.

With both pRL-TK and pRL-CMV as an internal control, the relative luciferase activity that represents the transcriptional activity of the CTGF/Hcs24 promoter was found to be markedly higher in HCS-2/8 than in HeLa. Similar studies were repeated 3 times with similar results. These results are consistent with the results of RT-PCR analyses shown in Fig. 1, and strongly suggest the observed strong promoter activity was a major cause of the highly active CTGF/Hcs24 expression in HCS-2/8 cells.

Repressive Effect of the CTGF/Hcs24 3′-UTR on Gene Expression in HCS-2/8—The CTGF/Hcs24 3′-UTR exhibits a *cis*-acting repressive effect on gene expression in monkey kidney COS-7 cells, as previously reported (*32*). However, its efficiency in chondrocytes or HCS-2/8 cells has not been addressed. Thus, we analyzed it, as a part of this study. One microgram of pGL3UTRS (shown as S) or pGL3UTRA (shown as AS) (Fig. 4A) was co-transfected with 1 μg of pRL-TK into HCS-2/8 cells. As positive and negative controls, pGL3-control and pGL3ΔP (Fig. 2A) were transfected instead of pGL3UTRS or pGL3UTRA, respectively. The relative expression levels were represented as relative measured luminescence values for firefly luciferase *versus Renilla* luciferase. As a result, the 3′-UTR of CTGF/Hcs24 gene was found to strongly repress the firefly luciferase gene expression in both sense and anti-sense forms. The repressive effect of the antisense 3′-UTR was stronger than that of the sense one. The sense 3′-UTR showed 62% repression, whereas the anti-sense 3′-UTR showed 82% repression (Fig. 4B). These findings for the 3′-UTR in HCS-2/8 cells are analogous to the previous results with COS-7 cells, indicating that the CTGF/Hcs24 3′-UTR is also functional in chondrocytic cells as a *cis*-acting repressor.

A Major Region in the Promoter Responsible for the Enhanced CTGF/Hcs24 Transcription in HCS-2/8 Cells—To explore the major region that is responsible for the strong gene expression in the CTGF/Hcs24 promoter in HCS-2/8 cells, deletion mutants were constructed from pTS589 (Fig. 5A). Each plasmid was transfected into HCS-2/8 following the established DNA transfection system, as described in a previous subsection. The luciferase activities of the deletion mutants are shown in Fig. 5B. There is not much difference among the luciferase activities of pTS589, pDS1, pDS2, and pDS3, but the difference between the activities of pDS3 and pDS4 is prominent in contrast to a previous report on unstimulated fibroblasts (*30*). Similar studies were repeated 3 times, with similar results. These results suggest major transcriptional element(s) that direct the observed constitutive expression of the CTGF/Hcs24 gene in HCS-2/8 cells in a 110 bp region, from –202 to –88, in the CTGF/Hcs24 promoter.

Response of the CTGF/Hcs24 Promoter to TGF-β1 Stimulation and Effects of Point Mutations in the TGF-β Response Element on the Transcription Activity of the CTGF/Hcs24 Promoter in HCS-2/8 Cells—To clarify whether or not the high basal activity of the CTGF/Hcs24 promoter in HCS-2/8 cells results from the TGF-β response element therein, point mutational analysis was performed. Four point mutations were introduced in the TGF-β response element (–157G→T, –155G→C, –153C→T, and –148G→T),

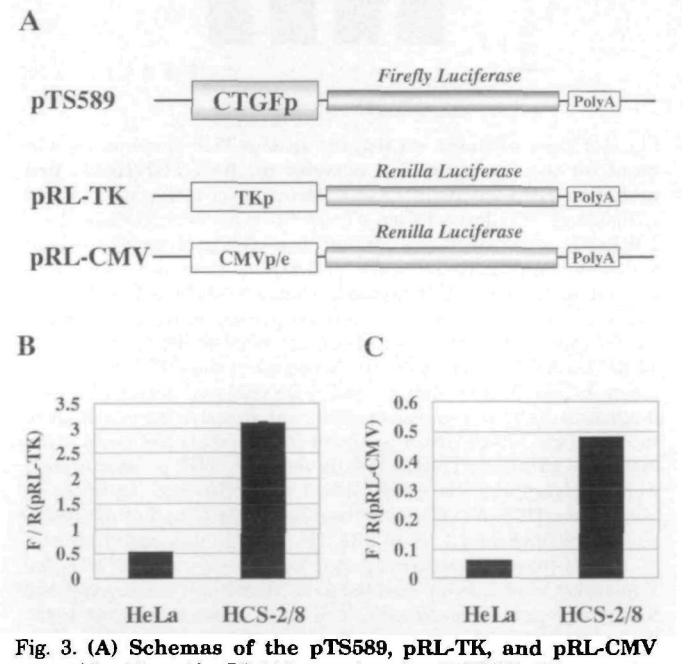

Fig. 3. **(A) Schemas of the pTS589, pRL-TK, and pRL-CMV plasmids.** Plasmid pTS-589 contains the CTGF/Hcs24 promoter linked to the upstream of a firefly luciferase reporter gene. As an internal control vector, pRL-TK or pRL-CMV (Promega) was used. CTGFp, CMVp/e, and TKp represent the promoters of human CTGF/Hcs24, CMV (with enhancer), and HSV-TK, respectively. All the polyadenylation signals originate in SV40. **(B)(C) Relative transcription activities of the CTGF/Hcs24 promoter in HCS-2/8 in comparison with HeLa.** One microgram of pTS589 and 1 μg of pRL-TK (B), or 1.5 μg of pTS589, and 0.5 μg of pRL-CMV (C) were co-transfected into HCS-2/8 or HeLa cells, and then luciferase activities were assayed. The expression levels are represented as relative luminescence values of firefly luciferase *versus Renilla* luciferase. The mean values of at least two assays are shown with error bars, which may not be visible when variations are very small.

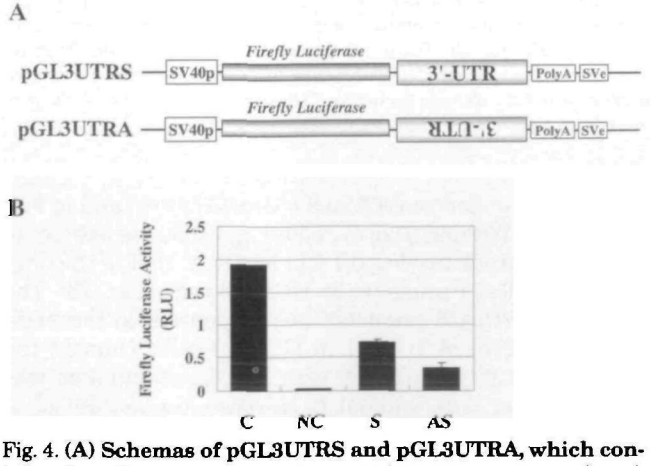

Fig. 4. **(A) Schemas of pGL3UTRS and pGL3UTRA, which contain *cis*-acting repressive element(s) on gene expression.** A sense or anti-sense form of the 3′-UTR of the CTGF/Hcs24 gene was linked to the downstream of a firefly luciferase reporter gene driven by an SV40 promoter, and termed pGL3UTRS or pGL3UTRA, respectively (*12*). **(B) Effect of the CTGF/Hcs24 3′-UTR on gene expression in HCS-2/8 cells.** Either 1 μg of pGL3UTRS (S) or 1 μg of pGL3UTRA (AS) was co-transfected with 1 μg of pRL-TK into HCS-2/8 cells. As positive (C) and negative controls (NC), pGL3-control and pGL3ΔP (Fig. 2A) were utilized. Expression levels are represented as relative measured luminescence values of firefly luciferase *versus Renilla* luciferase.

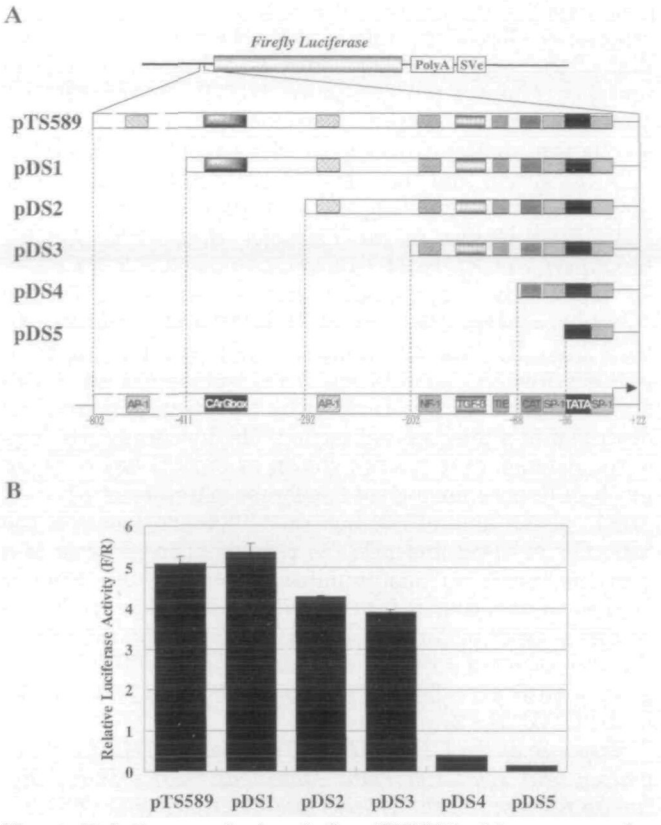

Fig. 5. **Deletion analysis of the CTGF/Hcs24 promoter-luciferase constructs in HCS-2/8.** (A) Schemas of the deletion mutants. Utilizing the CTGF/Hcs24 promoter segment in pTS589 as a PCR template, five mutants were constructed. Putative and experimentally proven *cis*-elements are shown in the boxes with a variety of shading, as described previously (*39*). Numbers below the major box indicate nucleotide numbers counted from the transcription initiation position. (B) Luciferase activities of the plasmids shown in panel (A). As an internal control vector, pRL-TK was used. DNA transfection was performed on a half scale with total DNA of 1 μg per sample, in which a mixture of 0.4 μg of pTS589 or its derivative, 0.4 μg of pRL-TK and 0.2 μg of carrier DNA was involved. Expression levels are represented as relative luminescence values of firefly luciferase *versus Renilla* luciferase. These results are representative of at least two independent sets of experiments.

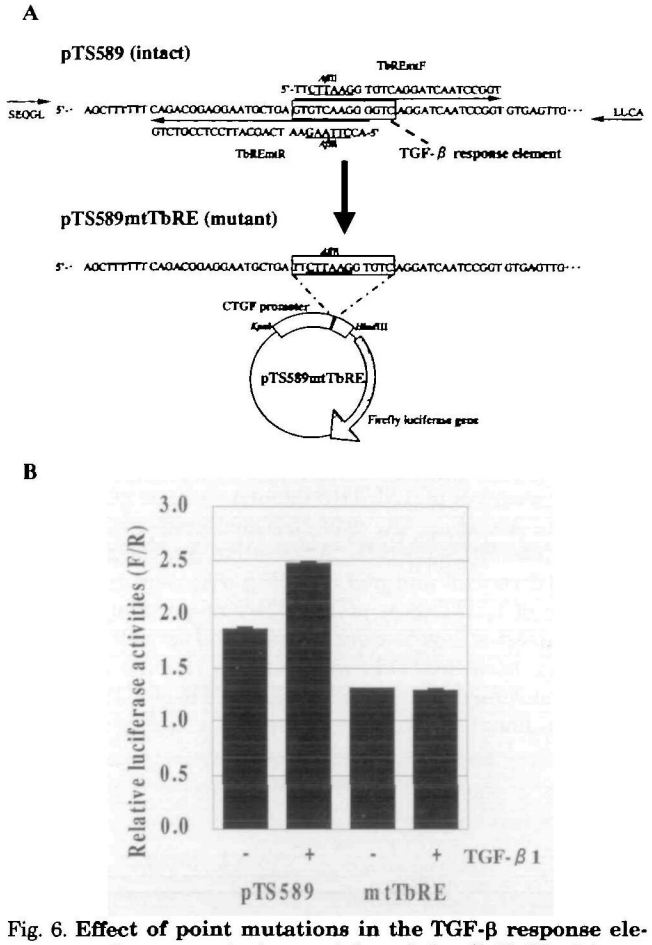

Fig. 6. **Effect of point mutations in the TGF-β response element on the transcription activity of the CTGF/Hcs24 promoter in HCS-2/8 cells.** (A) Construction of a mutant plasmid, pTS589mtTbRE, derived from pTS589. Two primers, TbREmtF and TbREmtR, to construct the mutant were designed, as shown here, and under "MATERIALS AND METHODS." Four mutations were introduced in the TGF-β response element (−157G→T, −155G→C, −153C→T, and −148G→T) and termed pTS589mtTbRE. The methods for constructing the mutant are described in detail under "MATERIALS AND METHODS." (B) Response of the CTGF/Hcs24 promoter on TGF-β1 stimulation in HCS-2/8 cells and the effect of point mutations in the TGF-β response element. Relative transcription activities of the CTGF/Hcs24 promoter in pTS589 or the mutant promoter in pTS589mtTbRE, with or without TGF-β1 stimulation, were analyzed with the established transfection and luciferase assay system. HCS-2/8 cells were transfected with 1 μg of pTS589 or pTS589mtTbRE and 1 μg of pRL-TK. TGF-β1 was added immediately after plasmid transfection at a final concentration of 10 ng/ml. Expression levels are represented as relative luminescence values of firefly luciferase *versus Renilla* luciferase. These results are representative of at least two independent sets of experiments.

as described under "MATERIALS AND METHODS," and in Fig. 6A in detail. Without TGF-β1 addition, luciferase activity of the mutant promoter was 0.7-fold less than that of the original CTGF/Hcs24 promoter in HCS-2/8 cells (Fig. 6B). The intact CTGF/Hcs24 promoter was responsive to the addition of 10 ng/ml of TGF-β1 in HCS-2/8 cells. Namely, the luciferase activity of pTS589 with TGF-β1 stimulation was 1.3-fold higher than without it. However, the mutant plasmid pTS589mtTbRE showed no response to TGF-β1 at all (Fig. 6B). These results suggest that the TGF-β response element is one of the major elements that direct the observed constitutive expression of the CTGF/Hcs24 gene in HCS-2/8 cells. It was also indicated that the signal transduction pathway through the TGF-β response element is active and still responsive to further TGF-β1 stimulation in HCS-2/8 cells.

DISCUSSION

Under physiological conditions, strong expression of CTGF/Hcs24 is not observed ubiquitously. Unexceptionally in growth cartilage, CTGF/Hcs24 is strongly expressed only in the hypertrophic zone (*29*). However, very strong expression of CTGF/Hcs24 was observed in HCS-2/8, which shows characteristics of immature to pre-hypertrophic chondrocytes in cell culture (*35–38*). The mechanisms of this unusual mode of CTGF/Hcs24 expression have remained unexplored. In this study, to explore the regulatory mecha-

nism of CTGF/Hcs24 gene expression in HCS-2/8, we analyzed two known regulatory elements of the CTGF/Hcs24 gene utilizing luciferase reporter gene assay systems. One element is the promoter, an upstream regulatory element, and the other is the 3′-UTR, a downstream regulatory element. In consequence, we revealed that the strong expression of the CTGF/Hcs24 gene is mainly caused by the enhanced transcription from the CTGF/Hcs24 promoter in HCS-2/8.

In this study, we found stronger CTGF/Hcs24 mRNA expression in HCS-2/8 than in HeLa on RT-PCR (Fig. 1A). The following luciferase assays clarified that the observed strong gene expression of CTGF/Hcs24 in HCS-2/8 is not caused by the release of the repressive effect by the CTGF/Hcs24 3′-UTR (Fig. 4B) (*32*), but by the strong transcription activity of the CTGF/Hcs24 promoter (Fig. 3, B and C).

There are several considerable mechanisms that may cause such a high transcriptional level of the CTGF/Hcs24 promoter. Of note is that it has been reported that the CTGF/Hcs24 transcript is rapidly induced after treatment of TGF-β1 in human skin fibroblasts and NIH3T3 fibroblasts, which is conducted by a TGF-β response element in the CTGF/Hcs24 promoter (*30, 31*). In HCS-2/8 chondrocytes, not only TGF-β1 but also bone morphogenetic protein–2 (BMP-2), which are members of the TGF-β family, increased the CTGF/Hcs24 mRNA level (*29*).

Then, we went for a determinant of this observed strong promoter activity. Deletion mutant analysis revealed a major DNA segment of a 110 bp region, from –202 to –88, in the CTGF/Hcs24 promoter, which directs the observed strong promoter activity in HCS-2/8 (Fig. 5). Therein is the TGF-β response element, which was identified by Grotendorst *et al.* (*30*). In their study, although CTGF/Hcs24 was slightly expressed in NIH3T3 fibroblastic cells and human skin fibroblasts under normal conditions, it was drastically induced by TGF-β stimulation through the TGF-β response element. In HCS-2/8, however, a 110 bp region, from –202 to –88, which includes the TGF-β response element, is responsible for the constitutive CTGF/Hcs24 expression. Therefore it was suspected that HCS-2/8 might produce TGF-β by itself and cause autocrine stimulation, maintaining the observed strong basal CTGF/Hcs24 promoter activity in HCS-2/8.

Therefore, we checked the responsiveness of the CTGF/Hcs24 promoter by means of TGF-β1 stimulation (Fig. 6B). After the stimulation the promoter activity was enhanced, although the effect was not very drastic. Firstly, this result indicates the responsiveness of the CTGF/Hcs24 promoter to TGF-β1 not only in fibroblasts, but also in chondrocytic HCS-2/8. Secondly, this moderate effect of TGF-β1 on the CTGF/Hcs24 promoter in HCS-2/8 might reflect that endogenous TGF-β1 or another member of the TGF-β super family already produced from HCS-2/8 at a high level may cause autocrine stimulation to maintain the observed strong basal CTGF/Hcs24 promoter activity in HCS-2/8.

To obtain direct evidence that TGF-β is a major factor that maintains the CTGF/Hcs24 transcription at a high level through the TGF-β response element, we introduced point mutations into the element (Fig. 6A). According to a previous report by Grotendorst *et al.*, artificial point mutations in this element nearly completely prevented the strong promoter activity induced by TGF-β in fibroblasts. Also in HCS-2/8, the point mutations completely abolished the TGF-β responsiveness. Clearly, no response of the mutant CTGF/Hcs24 promoter to the stimulation by TGF-β1 was observed (Fig. 6B). This indicates that the observed responsiveness of the intact CTGF/Hcs24 promoter to the TGF-β stimulation was solely through the TGF-β response element. Furthermore, the basal transcription activity was also decreased by the point mutations, albeit to a moderate level (–30%). Overall, these findings indicate that the TGF-β response element is one of the active elements that enable the strong CTGF/Hcs24 transcription in HCS-2/8, whereas there should be other active enhancer(s) in the region from –202 to –88 which are also critical for the strong transcription of the CTGF/Hcs24 gene specific to HCS-2/8.

Transcription factor(s) that directly promote HCS-2/8- or chondrocyte-specific CTGF/Hcs24 transcription, or signal transduction pathways to the CTGF/Hcs24 promoter in these cell types have not been clarified yet. Recently, a few CTGF/Hcs24 transcription regulators were identified. Smad binding element was identified in the CTGF/Hcs24 promoter and Smads transmit TGF-β signaling through the element in fibroblasts (*43*). VEGF, which is also a well-known angiogenic factor, induces CTGF/Hcs24 through its receptors, KDR and Flt-1, and signaling molecule Akt (*44*). It would help answer these questions to find direct mediator(s) that promote the high transcription levels observed in this study, by means of molecular biological approaches, such as gel shift assaying and the South-western blotting technique.

The 3′-UTR may be still a possible element that regulates gene expression through chondrocyte differentiation. Although the initial discovery of the *cis*-acting repressive function of the 3′-UTR of the human CTGF/Hcs24 gene had already been made using COS-7 fibroblastic cells (*32*), effects in chondrocytic cells were confirmed for the first time in this study. Indeed, the repressive functionality of the 3′-UTR of the human CTGF/Hcs24 gene was observed in HCS-2/8 in a similar manner to in the case of COS-7. Accordingly, it is unlikely that the 3′-UTR plays a positive role in the strong expression of the CTGF/Hcs24 gene, at least in HCS-2/8. Recently, an 84 base minimal RNA element, which acts as a *cis*-acting element of structure-anchored repression (CAESAR), was identified at the junction of the coding region and the 3′-UTR of *ctgf* (*33*). It was demonstrated that the repressive effect of CAESAR is dependent on its mRNA secondary structure. Since CAESAR did not affect the steady-state mRNA level, possible functioning stages of CAESAR were restricted to the mRNA export process and the protein translational stage. Release of the CAESAR-mediated repression of CTGF/Hcs24 expression may be a key for the mechanism of hypertrophic chondrocyte-specific expression of CTGF/Hcs24 in cartilage, fibrosis, and certain other types of oncogenesis, in addition to the observed high transcriptional activity.

In summary, it was indicated that the strong expression of the CTGF/Hcs24 gene in HCS-2/8 cells was mainly caused by high transcriptional activity of the CTGF/Hcs24 promoter. Undoubtedly, the TGF-β response element is one of the important elements, however, there should be other uncharacterized active element(s) in the CTGF/Hcs24 promoter for the constitutive gene expression of CTGF/Hcs24, which is specific to HCS-2/8 cells. Based on the present results, we are continuing our work to clarify the regulatory

mechanism of CTGF/Hcs24 gene expression that may conduct chondrogenesis and, occasionally, immortalization and transformation of chondrocytes.

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