

Cytokine Control of Interstitial Collagenase and Collagenase-3 Gene Expression in Human Chondrocytes*

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Human collagenase-3 expression, previously seen only in a breast tumor tissue, is here shown to be expressed in primary human chondrocytes derived from the joint tissue and in transformed human chondrocytes. Its mRNA is inducible by the inflammatory cytokines interleukin-1 β plus tumor necrosis factor- α , but not by phorbol 12-myristate 13-acetate and only slightly by the growth factors platelet-derived growth factor and epidermal growth factor. Human synovial fibroblasts, another prominent cell type in the joint tissue, do not produce collagenase-3 message. Expression of the murine collagenase, which is possibly the counterpart of human collagenase-3, is induced by interleukin-1 β plus tumor necrosis factor- α , and its full induction requires the presence of the transcription factor, c-FOS. This family of transcription factors also plays a role in induction of human collagenase-3, since it binds to the AP-1 site of this matrix metalloproteinase.

The matrix metalloproteinases (MMPs)¹ are a family of related enzymes that degrade the extracellular matrix. These enzymes operate during normal development in tissue differentiation and remodeling, but are also active under pathological conditions to cause inflammatory disease, degradation of bone and cartilage, as well as tumor metastasis (1–3). They are secreted as inactive latent forms, depend upon a zinc-binding active site, and are inhibited by chelating agents (4) and by specific proteins referred to as the tissue inhibitors of matrix metalloproteinases or TIMPs (5). In general, the MMPs show a certain degree of substrate specificity: the collagenases degrade collagen types I, II, and III; the gelatinases act on types IV, V, VII, and X collagens; while elastin and the stromelysins show a broader specificity, acting on proteoglycans, laminin, and fibronectin (4).

MMP levels are controlled by a variety of agents that include growth factors, *e.g.* epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF); the cytokines, particularly interleukin-1 β (IL-1) and tumor necrosis factor- α (TNF); and phorbol 12-myristate 13-

acetate (PMA), the protein kinase C activator. Glucocorticoids, retinoic acid, and transforming growth factor- β generally repress the induction of the MMPs (2–4). MMP transcription is controlled by *cis*-acting DNA elements located in the 5'-flanking sequence of their promoters, of which the two well known ones are the AP-1 site and the Ets-binding site, for responsiveness to cytokines, growth factors, and PMA (3). The AP-1 core consensus sequence, TGAGTCA (6), binds heterodimers of the FOS and JUN family of protooncogenes, and, with the exception of gelatinase A, this element is present in the promoters of all MMPs examined. Its unequivocal role in MMP gene expression was demonstrated in cell lines derived from c-FOS gene knockout mice, where the magnitude of induction of mouse collagenase and stromelysin-1 mRNA by growth factors was reduced considerably but restored to normal levels by the introduction of c-FOS into these cells (7).

The recently discovered human collagenase-3 (COL-3) is an important addition to the MMP family of proteins. Whereas, interstitial collagenase or COL-1 is observed frequently in most tissues and neutrophil collagenase or COL-2 occurs predominantly in neutrophils, COL-3 was localized to only human breast tumors (8). Given the substantial degree of cartilage collagen degradation in rheumatoid arthritis, and the relatively poor cleavage of type II collagen by COL-1, we have examined COL-3 expression in primary cells, both synovial fibroblasts and chondrocytes, derived from the joint tissue. Our results demonstrate that human COL-3 is not exclusive to breast tumor tissue but is expressed by primary and transformed chondrocytes and is inducible by IL-1 plus TNF. The cytokine induction of mouse collagenase, an enzyme closely related to human COL-3, is here shown to be dependent upon c-FOS. Results from our gel supershift experiments further indicate that the AP-1 site of human COL-3 is recognized by the FOS family of proteins including FRA-1, another member of the c-FOS family of transcription factors.

MATERIALS AND METHODS

Cells and Cell Lines—Primary human articular chondrocytes (HAC) and human synovial fibroblasts (HSF) were provided by Dr. David Woolley (University of Manchester). HACs were obtained by enzymic dispersion of the knee joint cartilage (9), rheumatoid synovial tissue was obtained by remedial synovectomy of the knees of patients with classic rheumatoid arthritis, and adherent synovium cell cultures were prepared as described (10). HACs were grown in DMEM/F12 supplemented with 0.29 mg/ml L-glutamine, 10 μ g/ml insulin, 5.5 μ g/ml transferrin, 6.7 ng/ml sodium selenite, and 50 μ g/ml ascorbate. HSFs were grown in DMEM/F12 supplemented with 3% L-glutamine and 10 ng/ml in tissue culture plates coated with 0.01% rat tail collagen. SW1353, a human chondrosarcoma cell line (ATCC HTB 94) and the 3T3 FOS (–/–) and FOS (+/+) cell line (from Dr. Bruce Spiegelman, Dana Farber Cancer Institute) were both cultured in DMEM. Culture medium was supplemented with 10% heat-inactivated fetal bovine serum.

DNA Probes—The human COL-3 cDNA probe is a 1.2-kb *Bam*HI-*Eco*RI fragment provided by Dr. Carlos Lopez-Otin (University of Oviedo, Madrid). The following probes were provided by Dr. Lynn

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¹ The abbreviations used are: MMP, matrix metalloproteinase; kb, kilobase pair(s); bp, base pair(s); PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1 β ; TNF, tumor necrosis factor- α ; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; AP-1, activator protein-1; RA, retinoic acid; HAC, human articular chondrocyte; HSF, human synovial fibroblast; COL-1, collagenase-1; COL-3, collagenase-3; STROM-1, stromelysin-1; DMEM, Dulbecco's modified Eagle's medium; MCP, human monocyte chemotactic protein.

Matrisian, Vanderbilt University: human stromelysin-1 (*STROM-1*), a 550-bp *Bam*HI-*Eco*RI fragment from the 3'-untranslated region; mouse collagenase, an 0.8-kb *Kpn*I-*Bam*HI fragment from the 3'-untranslated region; and human *COL-1*, a 640-bp *Clal*-*Bgl*II fragment. The glyceraldehyde-3-phosphate dehydrogenase probe was purchased from Clontech.

Northern Blot Analysis—RNA was prepared by the guanidinium isothiocyanate procedure and analyzed by electrophoresis in 1.0% agarose gels with 10 mM sodium phosphate buffer (pH 7.0), transferred to Hybond neutral membrane (Amersham), and hybridized with α -[³²P]dCTP probes prepared with a Random Primer Labeling Kit (Life Technologies, Inc.) to a specific activity of 10^8 – 10^9 cpm/ μ g DNA. Filters were hybridized, washed, exposed to x-ray film, and developed as described earlier (11).

Nuclear Extracts—Nuclear extracts were prepared by using the method of Mattila *et al.* (12). Briefly, 5×10^7 cells were washed with buffer A (10 mM HEPES pH 7.8, 15 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 500 μ g/ml Pefabloc SC from Boehringer Mannheim, 500 ng/ml leupeptin, and 700 ng/ml pepstatin) and resuspended in Buffer A containing 0.05% Nonidet P-40. The released nuclei were pelleted and resuspended in a final volume of 540 μ l of buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 μ g/ml Pefabloc SC, 500 ng/ml leupeptin, and 700 ng/ml pepstatin), to which 60 μ l of 3 M (NH₄)₂SO₄, pH 7.9, was added. The precipitated proteins were removed by centrifugation at 100,000 rpm in a Beckman TLA 100.2 rotor, and an equal volume of 3 M (NH₄)₂SO₄ was added to the supernatant. The precipitated proteins were collected by centrifugation at 100,000 rpm, suspended in 100 μ l of buffer C, and 10- μ l aliquots were frozen at -70°C until use. Protein concentrations were measured by the Bradford assay (13).

Electrophoretic Mobility Shift Assay—These assays were performed using the digoxigenin labeling kit (Boehringer Mannheim). 30 fmol of digoxigenin-labeled, double-stranded oligonucleotides were mixed with binding buffer and nuclear extract containing 10 μ g of total protein at room temperature for 20 min. For competition experiments, 4 pmol of unlabeled oligonucleotide was used, and their specific sequences are given in Fig. 3. Reactions were loaded onto 6% nondenaturing polyacrylamide (80:1 acrylamide:bisacrylamide) gels and electrophoresed at 120 V in $0.5 \times$ TBE (0.045 M Tris borate, 0.001 M EDTA) for 4 h at 4°C . Oligonucleotides were transferred electrophoretically to positively charged nylon membranes using a Hoefer Transphor apparatus and UV-cross-linked with a Stratalinker (Stratagene). Oligonucleotide mobility was detected with alkaline phosphatase-labeled anti-digoxigenin antibody and lumigen PPD (Boehringer Mannheim) substrate. Super-shifts were performed as gel shifts, with the addition of 5 μ g of specific antibody (Santa Cruz Biotechnology) to the oligonucleotide-extract reaction mixture.

RESULTS

Expression of COL-3 in Human Chondrocytes—Many MMP genes are induced by inflammatory cytokines, growth factors, and the tumor promoter PMA, and here their effect on the recently cloned human *COL-3* (8) was tested. High levels of *COL-1* are expressed by cells present in the human knee joint tissue; therefore, we chose to examine two different types of primary cells obtained from this source: *i.e.* chondrocytes, that are responsible for maintaining cartilage-specific matrix phenotype in normal joints, and synovial fibroblasts, cells lining the synovium of the joint. Both cell types are implicated in the destruction of the joint during rheumatoid arthritis (14). Primary human articular chondrocytes (HACs) were treated with IL-1 plus TNF, PDGF plus EGF, PMA, or retinoic acid (RA). RNA was isolated and analyzed by Northern blot analysis. When probes specific for human *STROM-1*, *COL-1*, or *COL-3* were used, mRNAs for *STROM-1* and *COL-1* were readily seen in the cytokine-treated primary cells, and a marked signal for *COL-3* was also noted. Whereas *STROM-1* was induced by only IL-1 plus TNF, *COL-1* was stimulated by IL-1 plus TNF, with growth factor treatment, and also with PMA. *COL-3* expression was increased markedly by the cytokines, to a small degree by the growth factors but not with PMA. Two species of *COL-3* mRNA were observed (Fig. 1A), and, although *STROM-1* and *COL-1* messages were seen in an overnight exposure of the film, the *COL-3* transcript levels required longer than a 24-h

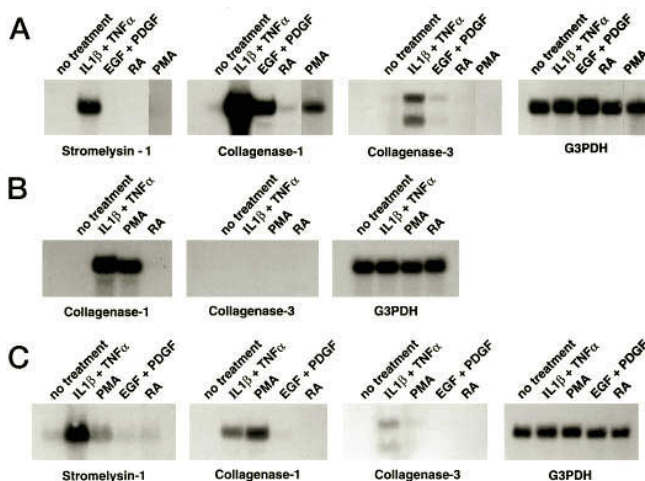


FIG. 1. Induction of MMP mRNA expression in primary and transformed human cell lines. Total RNA was isolated from human articular chondrocytes (A), human synovial fibroblasts (B), or human chondrosarcoma, SW1353 cells (C). Cells were treated overnight with PMA (100 nM), RA (10 μ M), IL-1 (30 ng/ml) plus TNF (50 ng/ml), or EGF plus PDGF (100 ng/ml each). 15 μ g of total RNA was loaded per lane, and blots were hybridized with specific DNA probes. A glyceral-3-phosphate dehydrogenase (*G3PDH*) probe was used to quantitate the amount of RNA loaded.

exposure.

Primary human synovial fibroblasts were also examined for *COL-1* and *COL-3* expression (Fig. 1B). As with human articular chondrocytes, *COL-1* message was readily detected upon IL-1 plus TNF or PMA treatment; but the two *COL-3* mRNA species could not be detected in these cytokine- or PMA-treated cells. In addition to these primary cells, a transformed chondrosarcoma cell line, SW1353 (Fig. 1C), was also examined, and in these the overall expression of the three MMP genes was similar in profile to the pattern from the primary chondrocytes, albeit at lower levels.

Collagenase mRNA Induction in Murine FOS (+/+) and FOS (-/-) Cell Lines—Human *COL-1* promoter induction by IL-1 (15, 16) and the induction of *STROM-1* message by TNF (17), both have been attributed to binding of proteins to the AP-1 site. Using the available murine *c-FOS*-deficient cells (7), we have further examined the role of the *c-FOS* family of transcription factors in the TNF and IL-1 induction of murine transin, the human stromelysin-1 equivalent, and murine collagenase. In these cells, the induction of transin and collagenase by EGF, PDGF, or PMA had already been shown to be *c-FOS*-dependent (7). The cell line derived from the *c-FOS* knockout mouse is designated *c-FOS*-deficient (-/-) cell line, and a cell line derived from a control wild-type mouse is designated wild type (+/+). In agreement with previous results (7), when both wild-type and *c-FOS*-deficient cell lines were treated with PMA, collagenase message was readily induced in wild-type (+/+) cells within an hour and increased over the 24-h period of induction (Fig. 2A). In contrast, in *FOS*-deficient (-/-) cells, only a very faint message was detected by 24 h. In our experiments with IL-1 plus TNF treatment of wild-type (*FOS* (+/+)) cells, murine collagenase message was induced within 2 h of treatment, but in *FOS*-deficient cells only a faint signal was obtained after 4–8 h of treatment (Fig. 2C). The behavior of stromelysin (transin), also an AP-1-dependent gene, showed a similar pattern of dependence on *c-FOS* for IL-1 plus TNF induction (Fig. 2B). It is clear from these results that *c-FOS* is required not only for PMA, but also for the IL-1 plus TNF response of these two murine MMP genes.

Gel-Shift Analysis to Examine the Role of *c-FOS* in the Induction of Human *COL-1*, *COL-3*, and *STROM-1* Genes—Het-

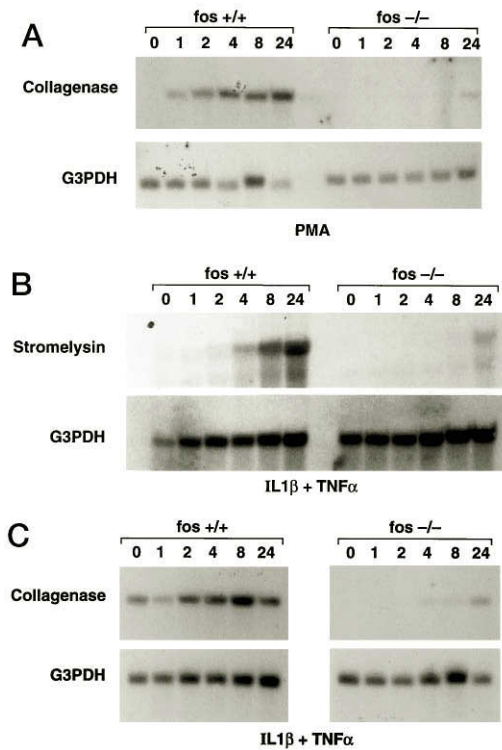


FIG. 2. Role of c-FOS in induction of MMP mRNA by IL-1 plus TNF. After 24-h serum starvation, c-FOS (+/+) and c-FOS (-/-) cell lines were treated for the indicated times with PMA (A) or IL-1 plus TNF (B and C). Total RNA was isolated, 10 μ g of total RNA was loaded per lane, and blots were analyzed with MMP-specific DNA probes. A glyceral-3-phosphate dehydrogenase (*G3PDH*) probe was used to quantitate the amount of RNA loaded.

erodimers of the FOS and JUN family of proteins are known to bind to the AP-1 promoter elements. The JUN family of transcription factors includes c-JUN, JUNB, and JUND, while the FOS family includes c-FOS, FOSB, FRA-1, and FRA-2 (18), and various combinations of these family members heterodimerize to bind to AP-1 sites with a consensus *tga(g/c)tca*. As shown above, c-FOS appears to participate in the mouse stromelysin and collagenase response to IL-1 plus TNF. Since the human COL-3 induction profile is clearly different from COL-1 or STROM-1 (Fig. 1), an examination of the composition of the FOS/JUN complexes that bind to the AP-1 sites of these human genes was conducted.

Nuclear extracts prepared from the chondrosarcoma or the primary human chondrocytes treated with the cytokines, or with PMA, were incubated with double-stranded AP-1 oligonucleotide sequences representing the core plus seven flanking nucleotides from the 5' and 3' end of each MMP promoter (Fig. 3). With extracts from SW1353 cells treated with PMA or with IL-1 plus TNF and AP-1 sites of human STROM-1, COL-1 and -3, or another MMP, gelatinase B (Gel B), the gel shift patterns produced appeared to be similar (Figs. 4, A and B). In each case, two specific bands were seen, and both bands could be competed with unlabeled oligonucleotide. Only the GELB bands were consistently fainter. Use of a mutant STROM-1 AP-1 site with a one nucleotide difference from the consensus markedly reduced the upper band and partially decreased the lower band, indicating specificity of the protein-DNA interactions. With an AP-1 site from the human monocyte chemotactic protein (MCP), a pattern quite unique and different from COL-1, COL-3, or STROM-1 AP-1 sites was obtained. Most likely, a considerable difference of the MCP AP-1 sequence from the consensus (Fig. 3) is reflected here. However, in extracts from both PMA- or IL-1 plus TNF-treated cells, the

Collagenase-1 AP-1	taa agc atg agt cag aca cct
Gelatinase B AP-1	tga ccc ctg agt cag cac ttg
Stromelysin AP-1	gca agg atg agt caa gct gcg
Mutant Strom AP-1	gca agg acg agt caa gct gcg
Collagenase-3 AP-1	taa gtg atg act cac cat tgc
huMTIIA AP-1	ccg caa gtg act cag cgc ggg
Mouse collagenase AP-1	aaa gtg gtg act cat cac tat
MCP AP-1	tcc tgc ttg act cgc ccc tct

FIG. 3. Sequences of AP-1 sites from various promoters. Nucleotides common to all AP-1 sites are in **bold** and represent the consensus sequence. The single base change made in the mutant stromelysin AP-1 site is indicated by an *asterisk*. Sequences are derived from the following sources: collagenase-1 AP-1 (15), collagenase-3 AP-1 (8), gelatinase B AP-1 (20), stromelysin-1 AP-1 (21), MCPAP-1 (22); metallothionein IIA AP-1 (23), and mouse collagenase AP-1 (Peter Angel, unpublished data). All sequences are from human genes except mouse collagenase.

patterns observed with the AP-1 sites of human STROM-1, COL-1, or GELB were strikingly similar to the AP-1 site of human COL-3. We have also tested nuclear extracts from primary human chondrocytes to compare the gel shift patterns of AP-1 sites of COL-1, COL-3, and mouse collagenase (Fig. 4C), and, as with the SW1353 extracts, here too, two shifted bands were observed. Also, the mouse collagenase AP-1 oligonucleotide gave the same pattern.

Since c-FOS plays an important role in PMA (7) and in the IL-1 plus TNF (this paper) induction of murine stromelysin and collagenase messages, and the corresponding human genes exhibit comparable AP-1 gel shift patterns, we have compared the AP-1 supershift patterns of COL-1 and COL-3 with c-FOS antibody. With antibody FOSX, cross-reactive with all known FOS proteins, the pattern of supershifted bands remained alike (Fig. 4D), and even with antibodies specific for FRA-1 and FRA-2, the patterns of supershifted bands remained identical. From these data it appears that c-FOS proteins bind to the human COL-3 AP-1 site in a manner similar to COL-1, or even stromelysin-1 and GELB, and, therefore, the differences in the expression profiles of the genes in the different cell types is likely not due to the FOS family of proteins.

DISCUSSION

Since the matrix metalloproteinases degrade a variety of extracellular matrix components, the synthesis of these enzymes is expected to be a highly controlled process. At the level of transcription, MMP control has been examined in different cell types in normal and pathological states. One pathological condition involving MMPs is rheumatoid arthritis, where these enzymes cause degradation of the cartilage. The behavior of the newly discovered human COL-3 in cartilage degradation was therefore of much interest even though the original source of its cDNA clone was from a breast carcinoma, and its expression in normal tissues was reportedly lacking (8). This observation was, however, not surprising since expression of most MMP genes is inducible, rather than constitutive. Therefore, we examined COL-3 induction in primary human chondrocytes, synovial fibroblasts, and a transformed human chondrosarcoma cell line by the inflammatory cytokines, IL-1 plus TNF. COL-3 expression was found in both primary and transformed chondrocytes but not in primary synovial fibroblasts. This is the first report of human COL-3 expression in nonmalignant cells in a disease state where cartilage degradation is commonly seen. Chondrocytes maintain the integrity of the cartilage in normal joints; but, in the arthritic joint, chondrocytes and synovial fibroblasts are influenced to change their properties by the action of inflammatory cytokines to begin secreting matrix-degrading enzymes and to eventually erode the joint tissue. Therefore, with this report, the relative roles of COL-3 and COL-1 in joint degradation becomes important.

In the report by Freije *et al.* (8), three collagenase mRNA species of 2, 2.5, and 3 kb were recorded, although the 2-kb

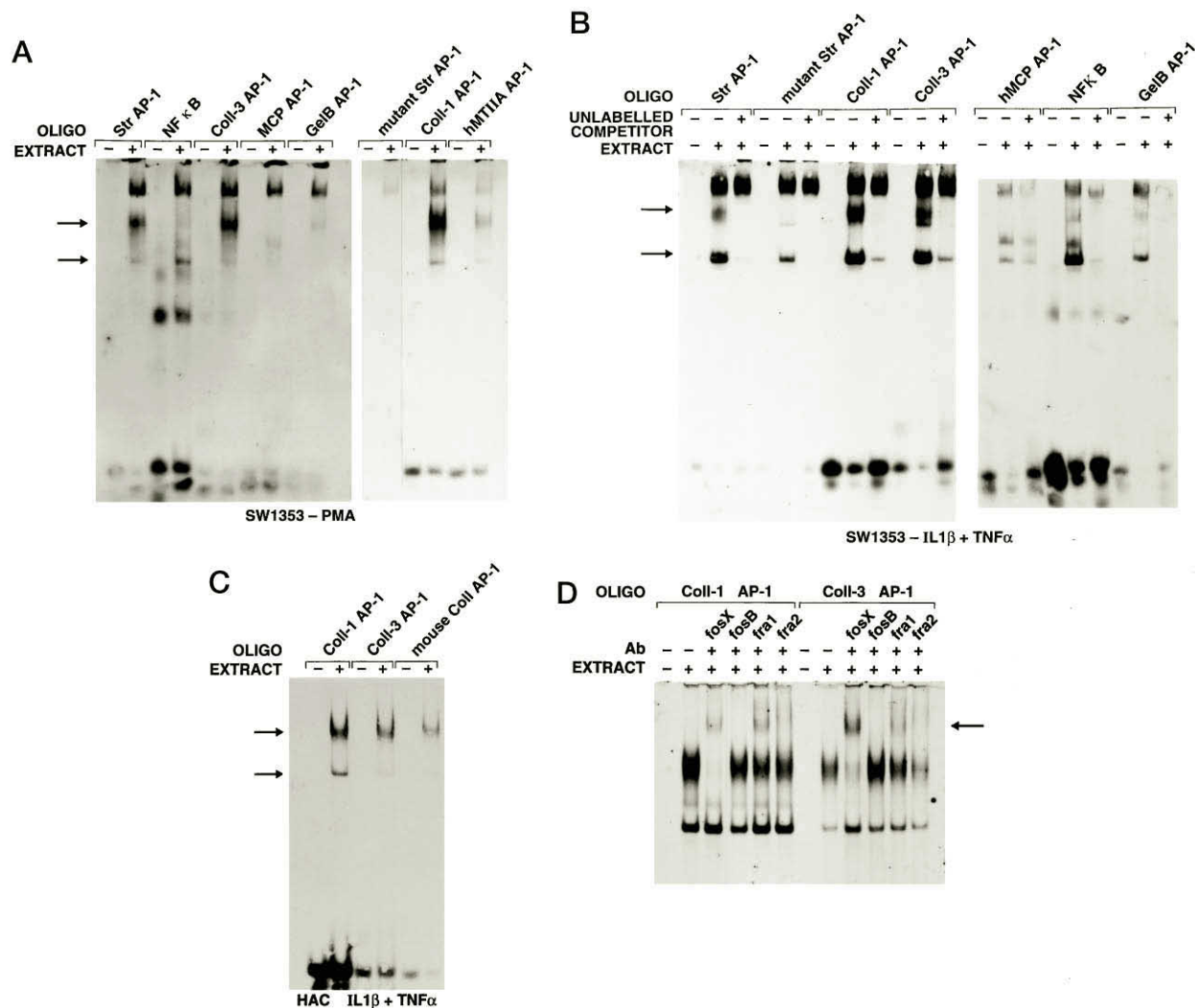


FIG. 4. Comparison of gel-shift and supershift patterns of AP-1 sites from various promoters. SW1353 (A, B, and D) or primary HACs (C) were serum-starved for 24 h, treated for 1–2 h with PMA (A) or IL-1 plus TNF (B, C, and D). Ten μ g of total protein were mixed with 30 fmol of digoxigenin-labeled double-stranded oligonucleotide (designated *OLIGO*), and samples were processed as described under “Materials and Methods.” In competition experiments, 4 pmol of unlabeled oligonucleotides were added simultaneously with label. In gel supershift experiments (D), 5 μ g of the indicated antibody were added, then extract, oligonucleotide, and antibody were allowed to react at 4 °C for 1 h. Sequences of AP-1 oligonucleotides used are given in Fig. 3. The sequence of the NF κ B-binding site used is: gat ctc aga ggg gac ttt ccg ag (12). Arrows indicate positions of specific shifted (A, B, C) or supershifted (D) bands.

band was weaker than the other two. In this report, with cytokine treatment, we could observe only two *COL-3* mRNA species in HACs or SW1353 cells. Most likely this is because of its low abundance or perhaps due to the reduced amount of total RNA loaded –40 μ g (8) versus 15 μ g in this report. Two species of *COL-3* message were also induced by EGF plus PDGF in confluent cultures of HACs, albeit weakly. *COL-1* and *STROM-1* were strongly induced by the cytokines, but the growth factors promoted only *COL-1* expression without *STROM-1* induction. Also, in primary chondrocytes, only *COL-1* was induced by PMA, although in the chondrosarcoma cells PMA was slightly more effective. These results quite remarkably demonstrate the unique cell-type specific responses to different inducers.

In mouse cells, stromelysin and collagenase induction by PMA and growth factors is c-FOS-dependent (7), and, in this report, we have shown that cytokine induction is also c-FOS-dependent. The message production was severely delayed in cell lines lacking c-FOS relative to induction within 1 or 2 h in wild-type cells (Fig. 2, B and C). The fact that there was any message for these MMPs at all may indicate that other FOS family members can substitute, albeit poorly, for c-FOS. It is

known that various combinations of FOS and JUN family members bind to identical sites with different affinities (19), and the binding of a lower affinity complex in this case may have resulted in less efficient transcription.

It was generally accepted that mouse collagenase was the murine counterpart of human COL-1, but a comparison of the amino acid sequences of human COL-3 and COL-1 with mouse collagenase has clearly shown the latter to be more closely related to human COL-3. Comparing the gel shift patterns of AP-1 regulatory control elements by nuclear extracts from chondrosarcoma cells (Fig. 4), no differences between human *COL-1* and *COL-3* were seen; similarly, with extracts from primary human chondrocytes, human *COL-3* and mouse collagenase AP-1 elements gave the same pattern. This similarity in pattern is observed despite the fact that human *COL-1* AP-1 site has a slightly different core sequence from human *COL-3* or the mouse collagenase elements (Fig. 3). A brief analysis with c-FOS antibodies showed that cross-reactive FOS antibody as well as specific antibodies for FRA-1, and to a lesser extent to FRA-2 gave supershifted bands (Fig. 4D) by recognizing proteins bound to the AP-1 oligonucleotides. From these data we have concluded that the set of c-FOS proteins binding

to the human *COL-3* AP-1 site are similar to the set that binds *COL-1*, *STROM-1*, or *GELB*.

We have in this investigation observed both cell type and inducer specific responses exhibited in primary human chondrocytes and synoviocytes. It is clear that the absence of *COL-3* expression in the synoviocyte is not due to a lack of responsiveness of this cell type to cytokines or PMA since *COL-1* expression is induced by them in these cells. It more likely indicates a *COL-3* promoter-specific response of its functional regions to transactivating nuclear proteins and the context in which they interact. In the core promoter region of the MMPs, in addition to AP-1, there are other well recognized regulatory elements of which the Ets-binding sites are conserved and known to interact with AP-1 (3). The human *COL-3* gene is located within a cluster of MMP genes on the long arm of chromosome 11 (24) and exploration of its regulatory sequence is likely to provide insights into the intriguing expression patterns observed with this gene.

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Addendum—While this paper was under consideration for publication, Mitchell (25) reported the cloning and expression of *COL-3* in chondrocytes from human osteoarthritic cartilage and its ability to cleave Type II collagen.

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