Nuclear factor binding to an AP-1 site is associated with the activation of pro- α 1(I)-collagen gene in dedifferentiating chondrocytes

Arto MÄÄTTÄ,* Virpi GLUMOFF,* Pia PAAKKONEN,* DeAnn LISKA,† Risto P. K. PENTTINEN* and Kati ELIMA*

*Department of Medical Biochemistry, University of Turku, SF-20520 Turku, Finland, and †Department of Biochemistry, University of Washington, Seattle, WA, U.S.A.

Isolated chondrocytes grown on plastic gradually lose their differentiated phenotype upon subculturing. This dedifferentiation is manifested by an altered production of extracellular-matrix molecules (ECM): e.g., the cartilage specific type II collagen is replaced by types I and III. We have studied the regulation of ECM gene expression in dedifferentiating human and murine fetal chondrocytes. Nuclear extracts from dedifferentiated cells, human fetal fibroblasts and 3T3 cells contained a protein that bound in an electrophoretic mobility shift assay to an AP-1 site in the first intron of the human α 1(I) collagen gene. This binding activity was not present in freshly isolated human or murine chondrocytes, which produced type II, but not type I, collagen mRNA in culture. Thus the binding activity was induced simultaneously with α 1(I)-collagen-gene

expression during dedifferentiation. The specific interaction was sensitive to dephosphorylation of the nuclear extract and to chemical modification of reduced cysteine residues. The AP-1 site we studied had previously been shown to be a positive transcriptional contributor in the first intron to the expression of the α 1(I) collagen gene. In transient transfections into dedifferentiating chondrocytes, an α 1(I) collagen expression plasmid carrying a mutated AP-1 site in the first intron resulted in three-times-lower reporter gene RNA levels than a plasmid carrying the respective functional AP-1 site. These data suggest that the AP-1 sequence and its respective *trans*-acting factors may play a role in the transcriptional regulation of the α 1(I) collagen gene during dedifferentiation of chondrocytes.

INTRODUCTION

Cellular differentiation is associated with major changes in the transcriptional activity of the genome, cell morphology and protein production. Transcriptional control of the differentiation and the extracellular matrix (ECM) surrounding the cells act reciprocally: matrix can maintain the phenotype of the cell, but it can also induce the differentiation process, which in turn may lead to expression of different matrix proteins [1].

Chondrocytes are highly differentiated mesenchymal cells that produce cartilaginous ECM, the main components of which are type II collagen, non-fibrillar collagens associated with type II collagen fibrils and proteoglycans [2,3]. Chondrocytes cultured in monolayer rapidly lose their differentiated phenotype and alter both their morphology and pattern of ECM protein expression towards a more fibroblastic one [4,5]. This dedifferentiation can also be achieved by either exposure of cells to bromodeoxyuridine (BrdU) [6] or retinoids [7]. Dedifferentiated chondrocytes cease to express type II collagen, whereas the expression of type I collagen is turned on [5]. This alteration provides a useful model to study the molecular mechanisms of down-regulation and activation of the genes for these proteins.

Several regulatory gene elements that participate in the control of pro- α 1(I)-collagen gene (COL1A1) have been described (for reviews, see [3,8]). The promoter of COL1A1 interacts both with positively acting transcription factors, such as a heterodimeric CCAAT-binding protein [9], and negative effectors, such as the recently purified inhibition factor ('IF') [10]. The role of sequences in the first intron for the expression of type I collagen genes is more controversial, but the intron is reported to contain both positive [11,12] and negative [13] regulatory elements; the best characterized is the positively acting AP-1-like motif [14].

Cultured primary chondrocytes and dedifferentiating chondro-

cytes provide a model to study the activation of the type I collagen genes and the repression of the type II collagen gene both at the chromatin level and at the level of individual protein binding sites in the promoter and enhancers. In the present study we have characterized changes in the binding of nuclear proteins to the AP-1 site in the first intron of COL1A1 [14]. In the dedifferentiated cells we find a strong binding that is not present in human and murine primary chondrocytes. In both dedifferentiated chondrocytes and human and murine fibroblasts the protein–DNA complex is sensitive to dephosphorylation of the nuclear extract and to changes in the reduction–oxidation state. Furthermore, transient transfections into dedifferentiating cells suggest that a functional AP-1 site in the first intron contributes to substantially higher expression than a mutated AP-1 site, which does not bind nuclear proteins.

MATERIALS AND METHODS

Chondrocyte isolation and cell culture

Human chondrocytes were isolated from 15–18-week fetuses from legal abortions and cultured as described [5]. The collection of samples and the experimental procedures have been approved by the Joint Ethics Committee of the Turku University Central Hospital and the University of Turku. For the isolation of fetal murine chondrocytes the same method was used, except that, in collagenase digestions, a 1.5 h instead of an overnight digestion with bacterial collagenase (Sigma) was used. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum.

Transient transfections and construction of mutated plasmids

Murine fetal chondrocytes from two independent isolations of

Abbreviations used: BrdU, bromodeoxyuridine; COL1A1, human pro- α 1(I)-collagen gene; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NEM, *N*-ethylmaleimide; UTR, untranslated region; hGH, human growth factor; 1 $\times$ SSC, 0.15 M NaCl/0.015 M sodium citrate; DTT, dithiothreitol.

chondrocytes were passaged to start dedifferentiation. Subconfluent cultures were transfected with calcium phosphate coprecipitation, essentially as described previously [13,14]. The following three plasmids were used: pColhGH carries a 0.8 kb $\alpha 1(I)$ collagen promoter-driven chimaeric gene consisting of the transcription start site, first exon and most of the first intron of the $\alpha 1(I)$ collagen gene fused to the fourth and the fifth exons of the human growth hormone (hGH) gene; pCol(M1-AP1)hGH, and pCol(M3-AP1)hGH differ from pColhGH by mutations in the first intron. The preparation of plasmid pCol(M1-AP1)hGH, in which the AP1 motif was mutated in such a way as to abolish binding of nuclear factors, has been previously described [14]. Site-directed mutagenesis of sequences flanking the AP1 motif, and subsequent preparation of the plasmid pCol(M3-AP1)hGH, were performed by the same procedure. The resultant mutation did not abolish the binding of nuclear factors, as assessed by gel-mobility-shift analysis using nuclear extracts prepared from fibroblasts (results not shown). All mutations were screened by dideoxy-DNA-sequencing method. Oligonucleotides were synthesized by the Howard Hughes Medical Institute Chemical Synthesis Facility, Seattle, WA, U.S.A. The structure of these oligonucleotides, indicating the mutations introduced into the S1 site, are as follows.

WT S1: 5'- GGGATGATTTCATAAGGAAAGATTG -3'
 M1-AP1: 5'- -----AG-AG----- -3'
 M3-AP1: 5'- -----CACCT----- -3'

An rous-sarcoma-virus promoter-driven β -galactosidase gene was co-transfected into each plate to control the transfection efficiency. After transfection, cells were chased for 48 h before the analysis of the expression levels.

RNA analysis

Confluent cells were harvested into guanidinium isothiocyanate and the total RNA was isolated by the CsCl-density-gradient centrifugation [15]. RNA (10 μ g) was fractionated in a 1% agarose/formaldehyde gel and blotted on to GeneScreen Plus membrane (New England Nuclear Corp., Boston, MA, U.S.A.). The filters were prehybridized and hybridized as described by the manufacturer, the most stringent washes were at 65 °C in 2 $\times$ SSC/1% SDS (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate). For the human pro $\alpha 1(I)$ collagen the probe pHCA1U [16], and for the human pro $\alpha 1(II)$ collagen the probe pHCAR3 [17] were used. The bound probe was detected by autoradiography at -70 °C using X-ray films (Kodak X-Omat) and intensifying screens. The probes were removed from the filters as recommended by the manufacturer. For the quantification of the hybridization signals the filters were probed with a cDNA probe for rat GAPDH [18]. RNA levels in the transient transfections were measured by slot-blot hybridization. RNA samples were applied on to GeneScreen Plus membrane (New England Nuclear) using a vacuum manifold (Schleicher and Schuell) and hGH gene mRNA (the reporter gene in transfection plasmids) was detected using a 0.68 kb *HindIII/Pvu II* fragment covering the fourth and fifth exons of the gene. The specificity of the probe and the hybridization conditions were confirmed by simultaneous hybridization of a Northern blot, including RNA sample from Rat-1 fibroblasts that had been stably transfected with a plasmid carrying the same hGH reporter gene driven by $\alpha 1(I)$ collagen promoter (results not shown). The Northern and slot blots were quantified using a laser densitometer (LKB Ultrascan), and the densitometric values were corrected with those obtained with the reference [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] probe.

Nuclear extracts

Nuclear extracts were prepared from confluent cell cultures as described [19,20] and dialysed against a buffer containing 12 mM Hepes, pH 7.9, 100 mM KCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 20% (v/v) glycerol and 1 mM phenylmethanesulphonyl fluoride. The protein concentrations were determined [21] and the extracts were stored in small aliquots at -70 °C. The small-scale procedure [20] was used to prepare nuclear extracts from the primary human chondrocytes and from the subcultured dedifferentiated cells. Other extracts were prepared using the standard large-scale method [19], and human skin fibroblast nuclear extract was prepared by both methods, as indicated in the Figure legends. Both large-scale and small-scale methods produced approximately equally active extracts, although the small-scale preparations contained slightly more non-specific proteins (A. Määttä, unpublished work).

Oligonucleotides

The oligonucleotides used in electrophoretic-mobility-shift assays were synthesized using an automated DNA synthesizer (Applied Biosystems). Each oligonucleotide (Figure 1b below) was annealed to its complementary strand as described in [14]. Double-stranded oligonucleotides were labelled with [γ -³²P]ATP using T4 polynucleotide kinase (Boehringer Mannheim) purified by PAGE, eluted from gel slices in 0.3 M potassium acetate/1 mM EDTA and precipitated with ethanol.

Electrophoretic-mobility-shift assays

Gel-retardation experiments of labelled oligonucleotide probes (10000 c.p.m.) and nuclear extracts were carried out as described previously [14,22]. Poly [d(I·C)] (2 μ g) (Boehringer Mannheim) was used as a non-specific competitor in all experiments. When indicated in the Figure legends, unlabelled double-stranded oligonucleotides were used to verify specific interactions. In some experiments the nuclear extracts were treated with 2–10 mM *N*-ethylmaleimide (NEM) (Sigma), 1–4 mM DTT or 0.5 unit of alkaline phosphatase (Boehringer Mannheim) 5 or 10 min before addition of the probe. The bound complexes were resolved from the free probe by electrophoresis in native 4.5%-polyacrylamide gel in 50 mM Tris/400 mM glycine buffer, pH 8.5, and detected by autoradiography of dried gels using intensifying screens.

RESULTS

Nuclear extracts were prepared from freshly isolated chondrocytes and from cells that had undergone a phenotypic switch from chondrocytes to dedifferentiated cells by passaging several times in culture. Dedifferentiated cells had a fibroblast-like morphology, in contrast with the cubical chondrocyte morphology of the cells in the primary cultures. We confirmed the phenotypic change by RNA analysis. Total RNA was isolated at the same time points as the nuclear extracts were prepared, and aliquots were blotted and sequentially hybridized with cDNA probes corresponding to human pro $\alpha 1(II)$ [17] and human pro $\alpha 1(I)$ collagens [16]. Densitometric quantification of the hybridization signals confirmed our previous results [5] on the dedifferentiation. The mRNA for type II collagen decreased to minimum (at least 60-fold) and the mRNA for pro $\alpha 1(I)$ collagen increased over 5-fold (result not shown).

We utilized double-stranded oligonucleotides corresponding to the previously described binding sites of an AP-1-like protein in the COL1A1 gene and nuclear extracts from chondrocytes, fibroblasts and dedifferentiated chondrocytes to elucidate possible changes in the regulatory protein-DNA interactions that

Figure 1 Localization and sequences of the studied nuclear-protein-binding sites

(a) The AP-1 related sequences S1 [14] and T1 are located in the first intron and in the 3' UTR of the COL1A1 gene respectively. The sizes of the exons are not drawn in scale. (b) Nucleotide sequences of the sense strands of the binding-site oligonucleotides. The core motifs are presented in **bold** letters. T2 contains the S1 sequence in an inverted orientation, and the GC-box oligonucleotide contains an Sp1 consensus sequence.

Figure 2 S1 and T1 sites interact with different nuclear proteins

(a) Gel-retardation assay with competing unlabelled binding-site oligonucleotides. Radiolabelled double-stranded S1 oligonucleotide was incubated with 10 µg of human fibroblast nuclear extract (large-scale) in the presence of 100-fold excess of double-stranded unlabelled competing oligonucleotide T1 (lane 1), T2 (lane 2), S1 (lane 3) or GC (lane 4) and the reaction mixtures were analysed on 4.5% native polyacrylamide gels. (b) Similar binding specificity is found in murine 3T3 fibroblast nuclear extracts. Nuclear extract (10 µg) of 3T3 fibroblasts was assayed for S1-binding proteins, with no oligonucleotide competitor (lane 1), 10-fold (lane 2) or 100-fold (lane 3) excess of double-stranded oligonucleotide S1 and 10-fold (lane 4) or 100-fold (lane 5) excess of T1.

associate with the dedifferentiation of chondrocytes. The locations of the S1 (intronic) and T1 (3' end) sequences in the COL1A1 gene are indicated in the Figure 1(a). The S1 probe was

Figure 3 A protein complex formed with S1 probe and nuclear extract from dedifferentiated chondrocytes is not found in human chondrocytes

Nuclear extracts from human fetal chondrocytes, 10 µg (labelled as C; lanes 1 and 3) or 14 µg from dedifferentiated chondrocytes (labelled as D; lanes 2 and 4) were incubated with radiolabelled double-stranded S1 (lanes 1 and 2) or T1 (lanes 3 and 4) oligonucleotide probes and the protein DNA complexes were resolved as described in the legend to Figure 2. The complexes are indicated with letters A–D according to their mobility.

described previously [14], and the T1 and T2 probes were chosen by combining our footprinting data (A. Määttä, V. Glumoff, P. Paakkonen, D. Liska, R. P. K. Penttinen and K. Elima, unpublished work) with that of Herget et al. [23]. The only difference between the intronic S1 [14] and the site in the 3' untranslated region (UTR) (called the 'tame' motif in [23] and T1 in the present study) is an extra C residue in the T1 motif. The T2 element actually contains the S1 sequence in an inverted orientation. We have previously described a 302 bp human sequence that includes the T1 and T2 sequences and corresponds to the rat 'tame' probe [23]. This fragment recognizes specifically a nuclear protein in fibroblasts and HeLa cells, and this binding activity is not found in myeloid cells [22]. Likewise, specific binding to intronic S1 site is not present in lymphoid Manca cell line [14]. The cell-specific protein binding obtained with the 302 bp insert [22] was also demonstrated with a synthetic double-stranded T1 oligonucleotide (results not shown). To exclude the possibility that the T1- and the S1 binding proteins were actually the same, we conducted competition assays with an excess of unlabelled specific or non-specific competitor oligonucleotides. Only the double-stranded S1 oligonucleotide was able to compete with the specific S1 binding, even in the presence of a 100-fold molar excess of either cold T1, T2 or GC-box (Sp1 consensus) oligonucleotides (Figure 2). This specificity was demonstrated with both human skin fibroblasts (Figure 2a) and murine 3T3 cells (Figure 2b). Similar results were obtained when labelled T1 or T2 oligonucleotides were competed for with the same repertoire of unlabelled competitors (results not shown). Thus we conclude

Figure 4 S1 binding complex A is absent in murine chondrocytes but present in 3T3 cells and human fibroblasts

(a) S1 probe was bound to 14 μ g of murine chondrocyte nuclear extract (MC, lane 1) or 10 μ g of the nuclear extract from 3T3 cells (lane 2). The complexes are indicated as in Figure 3. (b) The complex A with the nuclear extract from the dedifferentiated chondrocytes (lane 1) has a mobility similar to a complex with human fetal fibroblasts (lane 2); 5 μ g of the small-scale extracts were used in the binding assay.

that the AP-1-like S1-binding site in the first intron of the COL1A1 gene and the sites at the 3' end of the gene interact with different nuclear proteins.

Next we tested nuclear extracts from freshly isolated first-passage human chondrocytes and subcultured dedifferentiated chondrocytes for their ability to bind double-stranded S1 and T1-probes. Dedifferentiated chondrocytes exhibited a S1-binding activity that was not present in chondrocyte extracts (Figure 3, lanes 1 and 2). Hereafter this slowest-migrating complex is referred as to 'complex A'. In contrast, there was no dramatic difference between chondrocyte and dedifferentiated chondrocyte nuclear extracts in their T1-binding activity (Figure 3, lanes 3 and 4). These complexes had a mobility similar to that of the S1-probe-binding complex C (Figure 3, lanes 1 and 4), which was also present in both cell types, and presumably represents a non-specific protein. This is further supported by the finding that complex A was the only one readily inhibited by the unlabelled S1 competitor probe, indicating that the S1-binding complexes B and D could also be non-specific ones (results not shown). These results suggest that a S1-binding activity is induced upon dedifferentiation.

Similar experiments were conducted with murine fetal chondrocytes and NIH 3T3 fibroblasts. Regulatory regions of COL1A1 are very conserved between human and rodents [14,22], allowing the use of binding-site oligonucleotides derived from human gene with murine nuclear extracts. The murine cell lines exhibited the same difference in S1-binding nuclear proteins as human chondrocytes and dedifferentiated chondrocytes. Protein-DNA binding reactions with mouse chondrocytes did not contain the slow mobility complex present in 3T3 fibroblasts

Figure 5 The formation of the S1 binding complex A is abolished by *in vitro* dephosphorylation

Aliquots corresponding to 5 μ g of nuclear proteins from human chondrocytes (C) or dedifferentiated chondrocytes (D) were treated for 5 min with alkaline phosphatase in nuclear-extract storage buffer (pH 7.9) and assayed for S1-(lanes 1–4) and T1-(lanes 5–8) binding activity. Lanes 1 and 5, non-treated chondrocyte extract; lanes 3 and 7, phosphatase-treated chondrocyte extract; lanes 2 and 6, non-treated extract from dedifferentiated cells; lanes 4 and 8, phosphatase-treated extract from dedifferentiated cells.

(Figure 4a, lanes 1 and 2). However, a slight difference in the mobilities of faster-migrating, non-specific complexes B to D was observed between human and mouse chondrocytes. It seems that the protein(s) participating in the complex A are not present or active in the primary chondrocytes, regardless of their origin. Further assays revealed that the specific complex A of the nuclear protein from dedifferentiated human chondrocytes had a mobility similar to a complex formed with human fetal fibroblast nuclear extract (Figure 4b, lanes 1 and 2). This indicates most likely that the nuclei of the dedifferentiated chondrocytes have acquired the S1-binding activity present in fibroblasts that synthesize high amounts of type I collagen.

Short incubation of the nuclear extract from dedifferentiated chondrocytes and 3T3 cells with alkaline phosphatase abolished complex A without affecting the complexes with higher mobility (Figure 5, lanes 2 and 4; results of 3T3 experiments not shown). This was observed after incubation at both +37 °C and at room temperature. Incubation of the control reactions (without phosphatase treatment) at +37 °C had no effect on the formation of the complex A. Furthermore, we tested the effect on phosphatase together with different proteinase inhibitors (phenylmethanesulphonyl fluoride or aprotinin) to exclude the possibility that the phosphatase treatment could actually activate nuclear proteinases or that the phosphatase preparation used could involve contaminant proteinases. In all cases phosphatase treatment inhibited the interaction (results not shown).

Recently, the reduction of SH groups in cysteine residues has been implicated in the regulation of several transcription factors [24,25,26]. We looked for the formation of the complex A in the

Figure 6 Alkylation of SH groups inhibits complex A formation

(a) Binding reactions of the S1 probe and 8 μ g of 3T3 nuclear extract were modified by addition of DTT (lane 2, 1 mM; lane 3, 4 mM) or NEM (lane 4, 3 mM; lane 5, 6 mM). Lane 1, non-treated control. (b) NEM treatment of 2 μ g of nuclear extract from dedifferentiated chondrocytes (lane 2, 3 mM) and control reaction (lane 1).

Table 1 RNA levels in transient transfections into dedifferentiating chondrocytes

Plasmids carrying the promoter and the first intron of $\alpha 1(I)$ collagen and hGH gene as a reporter were transfected as a calcium phosphate co-precipitate into dedifferentiating murine fetal chondrocytes as described in [13,14]. The cells were derived from two independent isolations and culturings of chondrocytes. An RSV-promoter-driven β -galactosidase plasmid was co-transfected to evaluate the transfection efficiency between different plates. The plasmid pCol hGH has intact intronic sequence, it contains COL1A1 5' end sequences from +804 to -1442. Plasmid pCol(M1-AP1)hGH is the same as pCol-hGH, except for a mutation in the S1 element. The M1-AP1 sequence no longer binds nuclear proteins. The plasmid pCol(M3-AP1)hGH carries a mutation in the region flanking the S1-AP-1 site. This alteration does not affect protein-DNA interaction of the S1 site. RNA levels were measured by slot-blot hybridization, the values were quantified by laser densitometry and corrected for the β -galactosidase expression. The mean of values from the pCol-hGH transfections was normalized to 1.00.

Plasmid	n	Relative hGH RNA levels (mean $\pm$ SEM)
pCol-hGH	5	1.00 $\pm$ 0.30
pCol (M3-AP1) hGH	9	0.81 $\pm$ 0.20
pCol (M1-AP1) hGH	9	0.31 $\pm$ 0.04

situations where we modified the fibroblast or dedifferentiated chondrocyte nuclear extract by either reduction with DTT or alkylation of the SH-groups with NEM. DTT treatment had a

positive effect on the formation of complex A (Figure 6a, lanes 1–3). This suggests that a portion of the S1-binding-protein pool is in an oxidized form and reduction increases the amount of the proteins capable of the formation of complex A. However, the complex was not formed after DTT treatment of nuclear extracts of first passage chondrocytes (results not shown). NEM treatment, on the other hand, effectively prevented the complex A formation in reactions with extracts from both fibroblasts (Figure 6a, lanes 4–6) and dedifferentiated chondrocytes (Figure 6b, lane 2). This implies that reduced SH groups are needed for the binding of the complex A forming nuclear protein to the S1 site. The complex A formation was also sensitive to an increase in the KCl concentration from 400 mM to 800 mM (results not shown).

Further support for the role of the intronic AP-1 site was gained in transient transfections into dedifferentiating cells. Plasmids carrying either an intact S1 site or a mutated non-functional site [14] in chimaeric genes, in which the $\alpha 1(I)$ collagen promoter/first intron cassette drives hGH reporter gene, were transfected as a calcium phosphate co-precipitate, and the levels of hGH mRNA were subsequently measured in the dedifferentiated chondrocytes. The hGH mRNA values from the plasmid pCol(M1-AP1)hGH (the mutated S1 site that does not bind nuclear proteins) were approximately 31% of the values from the control transfections with the plasmid pColhGH (Table 1). Furthermore, values from transfections with the plasmid pCol(M3-AP1)hGH, which carries a mutation in the sequence flanking the S1-AP site that does not affect protein binding, were almost the same as with control plasmid pCol hGH. This suggests that an intact S1-AP1 site in the first intron of $\alpha 1(I)$ collagen gene might result in a higher activity of the $\alpha 1(I)$ promoter in dedifferentiating chondrocytes than a mutated S1 site that does not bind nuclear proteins.

DISCUSSION

We have investigated the protein-DNA interactions involved in the activation of COL1A1 during chondrocyte dedifferentiation. During repeated passages in cell culture chondrocytes lose their differentiated morphology and switch their pattern of extracellular matrix protein expression to a more fibroblastic one. Specifically, the expression of type II collagen ceases and the production of type I collagen commences [5]. We first chose to characterize changes in the protein-DNA interactions that putatively could participate in the activation of COL1A1. We felt that the recently described AP-1 like motif in the first intron of the gene could be one of the key regulators for the following reasons. First, a point mutation that inhibits the protein-DNA interaction in this motif abolished the net positive effect of the intron on the expression of a reporter gene in transient transfection assays [14]. Secondly, the extent of the transcriptional activation by the intact motif [14] was of about the same magnitude as the activation we had observed in the dedifferentiation of chondrocytes. Furthermore, when a fragment containing S1-motif (+292–+670; +1 being the transcription start site) was introduced 5' to the COL1A1 promoter a 2–5-fold transcriptional enhancement was observed [27]. Finally, we found that the intronic AP1 site had a nucleotide sequence closely resembling that of nuclear-factor-binding sites at the 3' UTR of the rat pro- $\alpha 1(I)$ -collagen gene [23]. This binding activity at the 3' end of the gene was co-ordinately regulated with the pro- $\alpha 1(I)$ -collagen transcripts in myoblasts; the binding activity was absent in the differentiated myotubes and present only in the myoblasts transcribing type I collagen genes [23]. So we also wanted to find out if the intronic and 3' UTR located binding sites interact with the same protein.

The utilization of small-scale nuclear extracts and gel-retardation assays with synthetic double-stranded oligonucleotides revealed that both human and murine chondrocytes lack a nuclear protein (or a protein complex) which selectively interacts with an AP-1-like element in the first intron of the COL1A1 gene. This binding activity (complex A) is, however, found in the nuclei of the dedifferentiated human and murine chondrocytes, and has a mobility similar to that of a complex formed with nuclear extracts from human and murine fetal fibroblasts. The formation of complex A can be inhibited by phosphatase treatment of the extracts (Figure 5). Furthermore, the formation of the complex A is enhanced by reduction of free SH groups and inhibited by alkylation by NEM (Figure 6).

The AP-1 sequence element binds homo- and hetero-dimers of the Fos/Jun superfamily. Additionally, Fos and Jun proteins are able to interact with other transcription factors of the ATF/CREB family [28]. Members of this family are expressed transiently after mitogenic, depolarizing or differentiation inducing stimuli, and are supposedly participating in the coupling of short-term cell-surface signals to changes in the cellular phenotype (for a review, see [29]).

The S1 motif differs from the consensus AP-1 site, TGA(C/G)TCA [30,31] by one nucleotide. This alteration of the central C/G to T had previously been shown to reduce the binding of c-Jun homodimer to less than 25% from wild-type binding [32]. However, it has been shown that the S1-binding site and its mutated form with the consensus AP-1 sequence bound equally well to nuclear proteins of fibroblasts [14]. When a four-base mutation was introduced to the core S1 site the specific binding was lost [14]. Furthermore, the T1 site used in this study has only one additional nucleotide compared to S1 site, but it does not bind to the same protein(s) as the S1 site (Figure 2). Thus it seems that the AP-1 motif is essential for the function of the S1 element. So far, we do not exactly know which particular heterodimer of the superfamily binds to the S1-site in the first intron of the COL1A1 gene. In HeLa nuclear extracts, antibodies against c-Fos inhibit the formation of S1-protein complex, indicating that c-Fos could be one of the participating proteins (D. Liska, unpublished work).

In the dedifferentiated chondrocytes an intact S1 motif resulted in about three times higher expression of a transfected plasmid than a mutated, non-functional S1 binding site (Table 1). Moreover, another mutation that does not abolish the protein DNA interaction resulted in approximately as high expression as the wild-type sequence. Thus it seems that when S1 activity is induced during the dedifferentiation, it is also needed to contribute to the elevated expression of the $\alpha 1(I)$ collagen gene. When the S1 site is mutated to not bind nuclear proteins, $\alpha 1(I)$ collagen promoter expression remains low in dedifferentiated chondrocytes. However, it is reasonable to assume that the S1 activity is not the only control mechanism that triggers the onset of $\alpha 1(I)$ collagen transcription. Dedifferentiation increases $\alpha 1(I)$ expression over 5-fold, which appears not to be fully reduced by point mutation at the S1 site.

Modification of the redox state has been implicated in the function of several regulatory nucleic acid-binding proteins. The binding activity of the Fos/Jun heterodimer was modified by reduction or oxidation of a single cysteine residue in the DNA-binding domains of the proteins [24]. Recently, the nuclear factor that stimulates the DNA binding of Fos and Jun via cysteine reduction has been purified and cloned [33,34]. Redox change seems to be a widely used mechanism for the activation of other transcription factors, e.g. NF- κ B [25] and Myb [26]. Our results are consistent with the redox regulation of AP-1-binding nuclear proteins. However, the lack of complex A-binding activity in the

chondrocytes is presumably not due to its presence solely in an oxidized form, because reduction with DTT could not introduce the complex in chondrocyte extracts.

The observation that a brief phosphatase treatment of the nuclear extract before the binding reaction abolishes the specific interactions suggests that a phosphorylated form of the protein(s) is (are) needed for the interaction or that a dephosphorylated form of an inhibitor prevents the interaction. The inhibition is unlikely to result from non-specific proteinase contamination of the phosphatase, since different proteinase inhibitors were used in the reactions. Phosphorylation has been attributed to both suppression and activation of the AP-1-binding proteins. Okadaic acid, an inhibitor of protein phosphatases and a blocker of myogenic differentiation, induced the AP-1-binding activity in C2C12 myoblasts [35]. This suggests that a similar phosphorylating-dephosphorylating mechanism might regulate the AP-1-binding complexes in myoblasts and fibroblasts. *In vitro* modulation of DNA binding by dephosphorylation has been described also in the case of the serum-responsive element binding p67^{SRF} [36,37]. However, DNA binding of the c-Jun is actually stimulated upon dephosphorylation [37], and phosphorylation of adjacent activator domain in H-Ras-expressing cells further increases the effect on transcription of the target genes [39]. Thus a plausible hypothesis would be that dephosphorylation regulates protein-DNA interaction in complex A by modifying the recently described IP-1, a dominant inhibitor of AP-1-DNA binding [40]. Only the non-phosphorylated form of IP-1 was found to inhibit the AP1-DNA interaction [40] in a manner similar to the inhibition we found.

The role of the 3' UTR located T1 site is more controversial. On the basis of the data presented here we suggest that the T1 site is not involved in the activation of the COL1A1 gene during chondrocyte dedifferentiation. It is noteworthy that our T1 site (Figure 2b) begins three nucleotides upstream to the site described in [23]. Herget and co-workers [23] found also that a nuclear extract from rat L6 myoblasts binds to a single-stranded (sense-strand) 'tame' oligonucleotide probe, whereas the single-stranded T1 oligonucleotide does not markedly bind nuclear HeLa or fibroblast proteins in our experiments (results not shown). Herget and co-workers used a catenated and inverted binding site that was combined from two different footprints [23]. Our probe is derived directly from the genomic sequence, which might, in part, explain the differences. The single-stranded-DNA-binding activity is possibly not related to the T1-binding activity. It might, in fact, actually represent RNA-binding proteins. We have recently demonstrated that an RNA probe transcribed from the same region of the conserved 3' UTR binds to several specific and non-specific nuclear proteins and at least one specific cytoplasmic protein (A. Määttä and R. P. K. Penttinen, unpublished work).

The absence of an AP-1-binding complex that recognizes the S1 element in the COL1A1 gene in chondrocytes and its presence in dedifferentiated cells and fibroblasts is of interest. A tissue-specific expression pattern of a transgene driven by the COL1A1 promoter has been observed without the intronic sequences [41]. This indicates that, albeit apparently tissue-specific, the S1-binding activity is perhaps more likely needed for the optimization of transcription than regulation of the tissue-specific collagen expression. AP-1-binding heterodimers are expressed transiently after mitogenic or differentiation-inducing stimuli [27,28]. The heterodimer corresponding to the complex A might thus enhance type I collagen synthesis in proliferating mesenchymal cells. On the other hand, it does not seem to be needed for high-level expression of cartilagenous extracellular matrix in chondrocytes. More studies are needed, however, to

elucidate the mechanisms controlling tissue-specific gene activation and the level of ECM gene expression.

The expert technical help of Mrs Merja Lakkisto, Mrs. Tuula Oivanen and Mrs. Marita Potila is greatly appreciated. Dr. Petri Auvinen and Dr. Eero Vuorio read the manuscript before its submission and successfully clarified several inconsistencies. This study was financially supported by the Cultural Foundation of Varsinais-Suomi (to A.M.), The Research and Science Foundation of Farnos Ltd. (to A.M.) and the Turku University Foundation.

REFERENCES

- Hynes, R. O. (1992) *Cell* **69**, 11–25
- von der Mark, K. (1986) *Rheumatology* **10**, 272–315
- Vuorio, E. and de Crombrughe, B. (1990) *Annu. Rev. Biochem.* **59**, 873–907
- Benya, P. D. and Brown, P. D. (1986) In *Articular Cartilage Biochemistry* (Kuettner, K. and Schleyerbach, R., eds.), pp. 219–233, Raven Press, New York
- Elima, K. and Vuorio, E. (1989) *FEBS Lett.* **258**, 195–198
- Saxe, S. A., Lukens, L. N. and Pawlowski, P. J. (1985) *J. Biol. Chem.* **260**, 3212–3218
- Yasui, N., Benya, P. D. and Nimni, M. E. (1986) *J. Biol. Chem.* **261**, 7997–8001
- Bornstein, P. and Sage, H. (1989) *Prog. Nucleic Acid Res. Mol. Biol.* **37**, 67–106
- Maity, S., Vuorio, T. and de Crombrughe, B. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5378–5382
- Karsenty, G., Ravazzolo, R. and de Crombrughe, B. (1991) *J. Biol. Chem.* **266**, 24842–24848
- Bornstein, P., McKay, J., Hoishima, J. K., Devarayalu, S. and Gelinas, R. E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8869–8873
- Rossouw, C. M. S., Vergeer, W. P., du Ploy, S. J., Bernard, M. P., Ramirez, F. and deWet, W. (1987) *J. Biol. Chem.* **262**, 15151–15157
- Bornstein, P., McKay, J., Liska, D. S., Apne, S. and Devarayalu, S. (1988) *Mol. Cell. Biol.* **3**, 4851–4859
- Liska, D. J., Slack, J. L. and Bornstein, P. (1990) *Cell Regul.* **1**, 487–498
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Mäkelä, J. K., Raassina, M., Virta, A. and Vuorio, E. (1988) *Nucleic Acids Res.* **16**, 349
- Elima, K., Vuorio, T. and Vuorio, E. (1987) *Nucleic Acids Res.* **15**, 9499–9504
- Fort, P., Marty, L., Piechaczyk, M., El Sabrouy, S., Dani, C., Jeanteur, P. and Planchard, J. M. (1985) *Nucleic Acids Res.* **13**, 1431–1442
- Ausbuel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.) (1989) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York
- Lee, K. A. W., Bindereif, A. and Green, M. R. (1988) *Gene Anal. Technol.* **5**, 22–31
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Määttä, A., Bornstein, P. and Penttinen, R. P. (1991) *FEBS Lett.* **279**, 9–13
- Herget, T., Burba, M., Schmoll, M., Zimmermann, K. and Starzinski-Powitz, A. (1989) *Mol. Cell. Biol.* **9**, 2828–2836
- Abate, C., Patel, L., Rauscher III, F. J. and Curran, T. (1990) *Science* **249**, 1157–1161
- Toledano, M. B. and Leonard, W. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4328–4322
- Guehmann, S., Vorbrueggen, G., Kalkbrenner, F. and Moelling, K. (1992) *Nucleic Acids Res.* **20**, 2279–2286
- Bornstein, P. and McKay, J. (1988) *J. Biol. Chem.* **263**, 1603–1606
- Kerppola, T. K. and Curran, T. (1991) *Curr. Opin. Struct. Biol.* **1**, 71–79
- Morgan, J. and Curran, T. (1991) *Annu. Rev. Neurosci.* **14**, 421–451
- Lee, W., Mitchell, P. and Tijan, R. (1987) *Cell* **49**, 741–752
- Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Ramsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) *Cell* **49**, 729–739
- Risse, G., Jooss, K., Neuberg, M., Brüller, H.-J. and Müller, R. (1989) *EMBO J.* **8**, 3825–3832
- Xanthoudakis, S. and Curran, T. (1992) *EMBO J.* **11**, 653–665
- Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E. and Curran, T. (1992) *EMBO J.* **11**, 3323–3335
- Park, K., Chung, M. and Kim, S.-J. (1992) *J. Biol. Chem.* **267**, 10810–10815
- Prywes, R., Ditta, A., Cromlish, J. A. and Roeder, R. G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7206–7210
- Ryan, W. A. Jr., Franza, B. R., Jr. and Gilman, M. Z. (1989) *EMBO J.* **8**, 1785–1792
- Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. and Hunter, T. (1991) *Cell* **64**, 573–584
- Smeal, T., Binétruy, B., Mercola, D., Birrer, M. and Karin, M. (1991) *Nature (London)* **354**, 494–496
- Auwerx, J. and Sassone-Corsi, P. (1991) *Cell* **64**, 983–993
- Slack, J. L., Liska, D. and Bornstein, P. (1991) *Mol. Cell. Biol.* **11**, 2066–2074