A fibroblast protein binds the 3'-untranslated region of pro- α 1(I) collagen mRNA

Arto MÄÄTTÄ* and Risto P. K. PENTTINEN

Department of Medical Biochemistry, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku, Finland

Post-transcriptional regulation of the expression of the pro α 1(I) chain of type I collagen (COL1A1) was studied by analysing cytoplasmic RNA-binding proteins and by transient transfections with collagen minigene plasmids. In this paper we present evidence for a factor from NIH 3T3 cells and human skin fibroblasts that interacts with the conserved 3'-untranslated region (UTR) of the shorter 4.8 kb mRNA species of the COL1A1 gene. The specificity of the interaction was confirmed by using (i) unlabelled specific and non-specific competitor RNAs and (ii) oligodeoxyribonucleotides annealed to the probe or used as single-stranded competitors. An antisense oligonucleotide annealed to the RNA probe near its 3'-terminus [20–42 nucleotides upstream of the first polyadenylation signal of the α 1(I) collagen mRNA] inhibited the binding, whereas other sense or

antisense oligonucleotides had no effect on the interaction. The binding was sensitive to alkylation of free SH groups but not to phosphatase treatment of the extracts. In u.v. cross-linking analysis this factor migrated as a single polypeptide chain of about 67 kDa, and was named α 1-RBF₆₇ (type I collagen α 1 chain RNA-binding factor). Dexamethasone treatment of fibroblasts, which is known to accelerate the turnover of COL1A1 mRNA, decreased the α 1-RBF₆₇ activity markedly as evaluated by gel-retardation and u.v. cross-linking assays. Transient transfections with plasmids carrying the α 1(I) collagen promoter and 3'-UTR sequences demonstrated that the 3'-UTR participates in the response to dexamethasone. Thus the loss of α 1-RBF₆₇ activity might be associated with decreased α 1(I) collagen mRNA levels after dexamethasone treatment.

INTRODUCTION

Type I collagen is a major structural protein of the extracellular matrix in most connective tissues. The gene for the α 1 chain of type I collagen (COL1A1) is transcribed to two mRNA species with molecular sizes of 4.8 kb and 5.8 kb, which differ only in the length of their 3'-untranslated region (UTR). In cell culture, collagen mRNA levels can be altered by external factors such as cytokines and hormones, or by changes in cell adhesion. Treatment with transforming growth factor β (TGF- β) stabilizes type I collagen mRNA in confluent 3T3 fibroblasts (Penttinen et al., 1988), and the effects of TGF- β can be blocked by γ -interferon at the post-transcriptional level (Kähäri et al., 1990). An increase in the mRNA half-life can also be seen after re-attachment of fibroblasts that had previously been grown in suspension (Dhawan et al., 1991). Steroid hormones, especially glucocorticoids, are used clinically to increase resistance to stress and for treatment of chronic inflammatory conditions such as rheumatoid arthritis or autoimmune diseases. One of the major side effects of chronic glucocorticoid administration is atrophy of skin, rupture of tendons or generalized osteoporosis (see Bornstein and Sage, 1989, for references). Glucocorticoids reduce the half-life of mRNA transcribed from the COL1A1 gene (Hämäläinen et al., 1985) which might be an important cause of the reduced collagen accumulation.

The regions in the COL1A1 gene or in the respective mRNAs responsible for post-transcriptional regulation have not yet been elucidated. Experimental evidence from other genes suggests that the 3'-UTR of mRNA might be important for mRNA stability. For example, destabilization of heat-shock protein 70 mRNA (Petersen and Lindquist, 1989) and *Xenopus* maternal Eg2 mRNA

(Bouvet et al., 1991), and stabilization of phosphoenolpyruvate carboxykinase mRNA by dexamethasone (Petersen et al., 1991) and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA by mitogens (Iwai et al., 1991), are mediated through their 3'-UTRs. Moreover, the 3'-UTR of the COL1A1 gene contains highly conserved regions (Mooslehner and Harbers, 1988; Herget et al., 1989; Määttä et al., 1991) for which no specific function is known. The whole 3'-UTR of the shorter 4.8 kb mRNA is highly conserved and was therefore chosen for investigation in the present study. Another conserved region lies around the latter polyadenylation site but is significantly shorter, about 100 bp long (Määttä et al., 1991).

The post-transcriptional regulation of gene expression is apparently carried out by recognition of specific sequences and/or secondary structures of the mRNA by certain proteins during its processing, transport to and storage in the cytoplasm. These sequences and the availability or binding affinity of the proteins distinguish between those mRNA molecules that will be stabilized and those to be degraded. At the moment, a few such regulatory proteins have been described; the best-known examples are those recognizing the AU-rich element (ARE) in many short-lived mRNAs (Malter, 1989; Brewer, 1991; Vakalopoulou et al. 1991; Bohjanen et al., 1991), and the iron-responsive-element-binding protein (for a review see Theil, 1990). Our goal is to characterize the mRNA-binding proteins and the sequences involved (i) in the selective stabilization or degradation of COL1A1 mRNA and, on the other hand, (ii) in the selection of the polyadenylation site. Elucidation of these mechanisms could also reveal the roles of these two transcripts. In this work we have characterized a specific cytoplasmic protein that binds a short segment of the 3'-UTR of the pro α 1(I) collagen mRNA.

Abbreviations used: α 1-RBF₆₇, α 1(I) collagen RNA-binding factor; FCS, fetal calf serum; hGH, human growth hormone; NEM, *N*-ethylmaleimide; UTR, untranslated region; TGF- β , transforming growth factor β ; DTT, dithiothreitol; ARE, AU-rich element.

* To whom correspondence should be addressed.

This protein is detected in NIH 3T3 cells and human skin fibroblasts and is regulated by glucocorticoids. In addition, results of transient transfections suggest that the 3'-UTR is needed for the dexamethasone-induced down-regulation of $\alpha 1(I)$ collagen mRNA levels.

MATERIALS AND METHODS

Protein extracts

Human skin fibroblasts and NIH 3T3 cells were cultured in bicarbonate-buffered Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Cytoplasmic extracts were prepared with the procedures designed for the isolation of nuclei (Shapiro et al., 1988; Lee et al., 1988). Cells were swollen in hypotonic buffer and disrupted by Dounce homogenization (Shapiro et al., 1988) or by repeated passages through a 25 G hypodermic needle (Lee et al., 1988). After pelleting of nuclei in a tabletop centrifuge, the cytoplasmic supernatant was cleared by recentrifugation for 30 min at 4 °C, dialysed against 12 mM Hepes, pH 7.9, 100 mM KCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 20% glycerol and 1 mM phenylmethanesulphonyl fluoride, frozen in aliquots and used as the cytoplasmic extract in the binding assays. Protein concentrations of the extracts were determined by the method of Bradford (1976). Protein extracts were dephosphorylated (Auwerx and Sassone-Corsi, 1991) by incubation with 0.1 to 0.5 unit of alkaline phosphatase (Boehringer Mannheim) at +37 °C for 5 min and subsequent quenching of the reaction by addition of NaH_2PO_4 to 50 μM . Control reactions were incubated with NaH_2PO_4 alone. For alkylation of free SH-groups of the cysteine residues, *N*-ethylmaleimide (NEM) was added up to 5 mM in the binding reactions 10 min before the addition of the probe.

Production of ^{32}P -labelled RNA probes

A 302 bp *TaqI*–*HindIII* DNA fragment (Figure 1a) coding for the 3'-UTR of the 4.8 kb COL1A1 mRNA species (Määttä et al., 1991) was subcloned from the cosmid clone CG 103 (Barsh et al., 1984) into a pGEM-1 (Promega) vector using standard methods of molecular biology. Plasmids for *in vitro* RNA synthesis were CsCl_2 -purified, linearized with an appropriate restriction enzyme (*HindIII* or *SspI* for sense strand probes or *EcoRI* for antisense transcripts) and extracted with phenol/chloroform (1:1, v/v). RNA probes were transcribed by commercial T7 or SP6 RNA polymerases (TransProbe, Pharmacia) as recommended by the manufacturer. Full-length transcripts were purified by electrophoresis in a 6% denaturing polyacrylamide gel, eluted from gel slices, phenol/chloroform-extracted and ethanol-precipitated. No remarkable differences were observed between the use of gel-purified or directly phenol/chloroform-extracted and ethanol-precipitated probes. Unlabelled competitor RNAs were transcribed *in vitro* with 1 mM UTP (Boehringer Mannheim) instead of [^{32}P]UTP from the same templates and from the fifth exon of the human growth hormone (hGH) gene subcloned into plasmid pGEM1. The homogeneity of the competitor RNA was evaluated on agarose/formaldehyde minigels.

RNA–protein interactions

In gel-retardation assays $(2\text{--}5) \times 10^4$ c.p.m. of the probe was incubated with the protein extracts for 10 min at room tem-

Figure 1 The 3'-UTR of the human pro $\alpha 1(I)$ collagen gene

(a) Partial restriction map of the 3'-UTR and templates of the RNA probes used. Open rectangle, end of the coding region; black vertical bars, polyadenylation sites. Highly conserved regions in the 3'-UTR (Määttä et al., 1991) are shown above the map. The location of the 0.3 kb *TaqI*–*HindIII* fragment (the PA1 probe) subcloned into plasmid pGEM1 for *in vitro* RNA synthesis is shown below the restriction map as a stippled box. (b) Schematic maps of the plasmids used in transient transfections. Hatched rectangle, the 2.3 kb $\alpha 1(I)$ collagen promoter/first exon/first intron cassette (nucleotides –804 to +1442); open rectangle, 0.68 kb *HindIII*–*PvuII* fragment containing hGH exons 4 and 5 (and in pEhGH including the 3' flanking sequences to the *NsiI* site); bold black line, COL1A1 3'-UTR and flanking sequences; black vertical bars, the polyadenylation signals.

perature in the presence of 200 ng/ μl *Escherichia coli* tRNA and 2 mg/ml heparin in a buffer containing 12 mM Hepes, pH 7.9, 15 mM KCl, 0.25 mM EDTA, 0.25 mM DTT, 5 mM MgCl_2 and 10% glycerol. In competition experiments unlabelled transcripts were incubated with the extracts for 10 min at room temperature prior to addition of the probe. The reactions were digested with 100 units of RNAaseT (Boehringer Mannheim) for 20 min at 20 °C. The bound probe was resolved from unbound digestion products by 4.5% PAGE without denaturants at 4 °C using a running buffer consisting of 50 mM Tris and 400 mM glycine, pH 8.0. The gels were dried and autoradiographed at –70 °C with intensifying screens. U.v. cross-linking of cytoplasmic proteins and labelled RNA probes was carried out essentially as described (Moore et al., 1988; Malter, 1989). Binding reactions were performed as described for the gel-retardation assays, exposed to u.v. light (254 nm) on ice at a distance of 4 cm for 20 min, and digested with a mixture of RNAase A and RNAase T for 30 min at 37 °C. Cross-linked proteins were resolved with electrophoresis in an 8% or 12.5% polyacrylamide gel in the presence of SDS along with ^{14}C -labelled molecular mass standards. Most gels were run in non-reducing conditions after we discovered that reduction did not alter the molecular masses of the complexes (results not shown). The gels were fixed in 20% trichloroacetic acid, dried and autoradiographed at –70 °C with intensifying screens.

Plasmid constructions and transient transfections

We used plasmids carrying hGH exons 4 and 5 driven by the COL1A1 promoter/enhancer (Bornstein and McKay, 1988). Plasmid pEhGH, also called pColhGH (a gift from Dr. Paul Bornstein, University of Washington, Seattle, WA, U.S.A.)

utilizes the 3'-UTR and polyadenylation signal of the hGH gene (Figure 1b), whereas plasmid pEhGH 2.2 contains the whole 3'-UTR of the COL1A1 gene starting from the same *TaqI* site as the PA1 probe and including both polyadenylation sites and about a further 0.9 kb 3'-flanking DNA (Figures 1a and 1b). The details of the construction and subcloning will be published elsewhere (A. Määttä, P. Bornstein and R. P. K. Penttinen, unpublished work; details available from the authors on request). The plasmids were purified by CsCl_2 centrifugation, and 10 μg of each was transfected into subconfluent 3T3 fibroblasts as a calcium phosphate co-precipitate. After 12–24 h the cells were washed several times with phosphate-buffered saline and chased for 24–48 h in the presence or absence of 1 μM dexamethasone (Sigma). The harvested cells were divided for the extraction of RNA and proteins. The transfection efficiency was estimated by co-transfection of 2 μg of Rous sarcoma virus- β -galactosidase plasmid to each plate and subsequent determination of β -galactosidase activity in cell extracts. The amount of hGH RNA was analysed by solution hybridization. A pGEM plasmid carrying the 0.15 kb hGH insert from the fifth exon was linearized and antisense riboprobes were synthesized and purified using the TransProbe T kit (Pharmacia). The protection assays for the RNA samples from transiently transfected cells were carried out as described (Bornstein and McKay, 1988). In short, 5 μg of total RNA was incubated with $(2-4) \times 10^5$ c.p.m. of hGH antisense riboprobe overnight at 45 °C. After digestion with RNases A and T and extraction with phenol/chloroform, the protected fragment was precipitated with ethanol using 4 μg of RNAase-free *E. coli* tRNA as a carrier and separated from the digestion products by electrophoresis in a 6% polyacrylamide/50% urea gel. After autoradiography the protected fragments were quantified with an LKB XL Gel Scan laser densitometer.

RESULTS

We used the PA1 probe (Figure 1) to analyse the cytoplasmic RNA-binding proteins of human skin fibroblasts and NIH 3T3 cells. The probe covers the highly conserved region of the 3'-UTR that is used during the synthesis of both the 5.8 kb and 4.8 kb COL1A1 mRNA species, and thus could have regulatory function for both RNAs. The predicted secondary structure of

Figure 2 Predicted secondary structure of the 3'-UTR of the shorter COL1A1 mRNA

The algorithm of Zuker and Stiegler (1981) was adopted to generate a potential secondary structure of the PA1 sense probe. The numbering of the bases proceeds in 5' to 3' direction and begins at the G residue in the *EcoRI* site in the map in Figure 1(a).

Figure 3 Fibroblast cytoplasmic proteins bind the PA1 RNA *in vitro*

PA1 probe (5×10^4 c.p.m.) was incubated with 10 μg of NIH 3T3 cell cytoplasmic protein extract in the presence of excess *E. coli* tRNA and heparin. The reaction products were digested with RNAase T and run in a 4.5% native PAGE gel. Lane 1, digested probe alone; lane 2, 10 μg of cytoplasmic extract from 3T3 cells; lanes 3–6, increasing (10-, 20-, 40- and 80-fold more than probe) concentrations of unlabelled hGH (a non-specific competitor RNA) incubated for 10 min with 10 μg of cytoplasmic extract of 3T3 cells prior the addition of PA1 probe; lanes 7–10, increasing (10-, 20-, 40- and 80-fold) concentrations of unlabelled PA1 RNA (a specific competitor) incubated for 10 min with 10 μg of cytoplasmic extract of 3T3 cells prior the addition of PA1 probe; lane 11, 10 μg of BSA instead of 3T3 extract; lane 12, binding reaction with 10 μg of 3T3 cytoplasmic protein extract digested with 15 μg of proteinase K prior to electrophoresis. The retarded complex is indicated by an arrow. Fast-migrating radioactive material in this and subsequent Figures represents free RNAase-digested probe.

the RNA probe (Figure 2) contains three distinct stems and loops and a long stretch of putatively single-stranded RNA. Protein–RNA interactions were studied by gel-retardation experiments. *E. coli* tRNA and heparin were added to the binding reactions as non-specific competitors, the reaction products were digested with RNAase T, and the complexes were resolved from unbound RNAase T fragments of the probe by non-denaturing polyacrylamide-gel electrophoresis. In these experiments a retarded RNA–protein complex was formed (indicated by an arrow in Figure 3). The shifted complex was not found if the reaction mixtures were treated with proteinase K prior to electrophoresis (Figure 3, lane 12) or if BSA was used instead of cytoplasmic extracts (Figure 3, lane 11). Complexes migrating with equal mobilities were formed from the probe and extracts of several different human skin fibroblast cultures (results not shown). The specificity of the RNA–protein interaction was studied in experiments with unlabelled specific and non-specific competitors. Increasing concentrations of non-specific competitor RNAs (e.g. RNA transcribed from the 5th exon of the hGH gene) added to the reactions did not inhibit complex formation (Figure 3, lanes 3–6). In contrast, the unlabelled PA1 RNA competed effectively for the binding (Figure 3, lanes 7–10).

The protein-binding site in the PA1 RNA was localized using competition experiments with both RNA transcripts and DNA oligonucleotides. First, we carried out binding assays using either the full-length PA1 probe or the 5'-terminal fragment of the PA1 probe (0.15 kb *TaqI*–*SspI* fragment) (Figure 1a). The results of these experiments indicated that the 5'-terminal fragment does not bind cytoplasmic proteins from human skin fibroblasts or NIH 3T3 cells (results not shown). Further series of experiments were conducted with cytoplasmic and nuclear proteins and the antisense PA1 transcript. This probe failed to recognize any proteins. However, protein–RNA complexes were again seen when the transcript from the 3'-terminal *SspI*–*HindIII* fragment of the PA1 probe was used (results not shown). Next we

Figure 4 The 5'-terminal fragment of the PA1 probe does not compete for binding

The binding reactions between the PA1 probe and the 3T3 cytoplasmic extract were carried out in the presence of increasing concentrations of unlabelled sense strand competitors (0.3 kb of PA1 full-length transcript or 0.15 kb of 5'-terminal *TaqI*-*SspI* transcript). All reactions included excess tRNA and heparin. The complexes were resolved from free RNAase T digestion products by electrophoresis in a 4.5% native gel. Lane 1, digested probe alone; lane 2, reaction without competitor transcripts; lanes 3–5, increasing (20-, 40- and 80-fold greater than probe) concentrations of the unlabelled PA1 RNA were incubated with 3T3 cytoplasmic extract for 10 min at room temperature prior to addition of 20000 c.p.m. of PA1 probe; lanes 6–8, increasing (40-, 80- and 160-fold) concentrations of the unlabelled 0.15 kb *TaqI*-*SspI* transcript PA1 RNA were added as a competitor.

performed competition experiments with increasing concentrations of unlabelled PA1 RNA, or with RNA from the 5'-terminal 0.15 kb *TaqI*-*SspI* fragment of PA1 (Figure 1a). Only the unlabelled full-length PA1 RNA competed with the labelled PA1 probe (Figure 4, lanes 3–5). Even high concentrations of the 0.15 kb *TaqI*-*SspI* transcript (Figure 4, lanes 6–8) did not markedly inhibit the interaction.

A more detailed mapping of the binding site was performed with PA1-specific sense and antisense oligonucleotides and with control DNA oligonucleotides (Table 1). The RNA probe was denatured by heating for 5 min at 80 °C and slowly allowed to renature with about 250 pmol of each oligonucleotide at a time. Thus antisense oligonucleotides (T1' and T2'; Table 1 and Figure 5) were annealed to the PA1 probe in order to prevent secondary structure formation during renaturation. Sense strand oligonucleotides (T1, T2 and P1) were used as single-stranded DNA competitors, as some RNA-binding proteins can also bind

single-stranded DNA (e.g. Smith et al., 1984). Three more control oligonucleotides (S1, S1' and P2) and, in some experiments, double-stranded oligonucleotides were further included to reveal possible non-specific effects. The 5'-terminal 0.15 kb *TaqI*-*SspI* fragment of the PA1 probe did not bind cytoplasmic proteins, in contrast to the 3'-terminal *SspI*-*HindIII* fragment (Figure 4), so we assumed that the protein-binding site is located in the 3'-half of the PA1 probe. For this reason, most of the oligonucleotides used (T1, T1', T2 and T2') were directed to this region. The T1' oligonucleotide, which is antisense to nucleotides 240–262 of the PA1 probe (Figure 5c) and maps 20–42 nucleotides upstream of the first polyadenylation site, effectively inhibited the formation of protein-RNA complexes between the PA1 probe and the cytoplasmic extracts of 3T3 cells (Figure 5a, lane 4) and human skin fibroblasts (Figure 5b, lane 4). Oligonucleotides T2 and T2' map close to oligonucleotides T1 and T1' (Figure 5c). However, they had no remarkable effect on PA1 protein-RNA complex formation (Figure 5a, lanes 5 and 6; Figure 5b, lanes 5 and 6) in gel-retardation experiments. Oligonucleotide P1, upstream of the T sites, and the control oligonucleotides had no effect on binding (Figure 5a, lanes 7–10). When oligonucleotides T1 and T1' were hybridized with each other into double-stranded DNA prior to their addition to the reaction mixtures, the resultant dsT (double-stranded) oligonucleotide did not interfere with the formation of the protein-RNA complexes (Figure 5a, lane 12). Taken together, the results of the gel-retardation experiments indicate that a specific cytoplasmic protein(s) binds the PA1 RNA probe. A 22-nucleotide-long region near the 3' end of the probe is a putative binding site, as a DNA oligonucleotide annealed to this site inhibits the formation of RNA-cytoplasmic-protein complex.

We continued the characterization of the protein-RNA binding mechanism and its regulation by dephosphorylation and modification of free cysteine residues in the cytoplasmic proteins. Both of these mechanisms have previously been implicated in the regulation of protein-RNA interactions (Hentze et al., 1989; Malter and Hong, 1991). Alkaline phosphatase treatment of the cytoplasmic extract had no effect on complex formation (Figure 6, lane 3), whereas alkylation of SH-groups with NEM effectively inhibited complex formation (Figure 6, lane 4). The involvement of thiol groups has been suggested to be a common feature of eukaryotic RNA-protein interactions (Hentze et al., 1989).

The u.v. cross-linking/label transfer method was used to evaluate the approximate molecular mass of the PA1 probe-binding protein(s). In this method, the probe and the bound proteins are covalently cross-linked (in the presence of excess tRNA and heparin) by irradiation with short-wave u.v. light.

Table 1 Oligonucleotides used in this study

The oligonucleotides were synthesized by an automated DNA synthesizer (Applied Biosystems) and used in competition experiments with and annealing to the PA1 riboprobe. The numbering of the bases corresponds to Figure 2.

Name	Sequence	Origin	Relation to PA1 probe
T1	5'-CAAAAGTGCATTCAACTTACCA	3'-UTR, COL1A1	Nucleotides 240–262
T1'	5'-TGGTAAGTTGAATGCACTTTTG	3'-UTR, COL1A1	Antisense to T1 site
T2	5'-CTCAAACCTAGTTTTATCTTTGACC	3'-UTR, COL1A1	Nucleotides 185–211
T2'	5'-GGTCAAAGATAAACTAAGTTTGAG	3'-UTR, COL1A1	Antisense to T2 site
P1	5'-TGGGAGACAATTCACATGG	3'-UTR, COL1A1	Nucleotides 139–159
P2	5'-TTGGGGTGTGTTGAGCATTGCC	3'-UTR, COL1A1	Control, outside the probe region
S1	5'-GCCGTGGGATGATTGATAAGGAAAGATTG	1st intron, COL1A1	Control, outside the probe region
S1'	5'-CAATCTTTCCTTATGAATCATCCACGGC	1st intron, COL1A1	Antisense to S1

Figure 5 Inhibition of PA1-protein interaction by a DNA oligonucleotide annealed to the PA1 probe

(a) The PA1 probe was denatured by heating and allowed to hybridize to the oligonucleotides (about 250 pmol) described in Table 1. Lane 1, probe alone. Lanes 2–12 were incubated with 10 μ g of 3T3 cell cytoplasmic extract: lane 2, probe denatured and renatured without DNA oligonucleotides; lane 3, excess of sense-strand oligonucleotide competitor T1 in the reaction; lane 4, probe annealed to the antisense oligonucleotide T1'; lane 5, excess of sense-strand oligonucleotide competitor T2 in the reaction; lane 6, probe annealed to the antisense oligonucleotide T2'; lane 7, excess of oligonucleotide S1 in the reaction; lane 8, excess of antisense oligonucleotide S1' in the reaction; lane 9, probe with an excess of sense strand oligonucleotide competitor P1; lane 10, excess of oligonucleotide P2 in the reaction; lanes 11 and 12, excess of double-strand oligonucleotides S and T respectively in the reactions. (b) Interference by oligonucleotides of the interaction between the PA1 probe and the human skin fibroblast cytoplasmic extract. See Table 1 for oligonucleotide design. Lanes 1 and 2, as in (a). Lane 3, oligonucleotide T1 as a competitor; lane 4, T1' annealed to the probe; lane 5, T2 as a competitor; lane 6, T2' annealed to the probe. (c) Nucleotide sequence of the 3'-half of the PA1 probe beginning from the *SspI* site. The triplicate AATAAA motif is in italics, the T1 sequence is double-underlined and the T2 sequence is underlined.

Unbound RNA and extra residues outside the binding site are digested with RNAses A and T, leaving several radioactive nucleotides covalently bound to the protein. SDS/PAGE of the digestion products followed by autoradiography gives estimated molecular masses of the proteins which, however, include a few kDa of RNA residues (Moore et al., 1988). The following control experiments were carried out: (i) incubation of the protein extracts with proteinase K prior to binding, (ii) electrophoresis of complexes without u.v. treatment, (iii) RNAase digestion of the probe before the complex formation, and (iv) incubation of the probe with a non-specific protein, BSA. No labelled proteins were observed in these control experiments (results not shown). NIH 3T3 cell cytoplasmic extracts contained a 67 kDa protein

Figure 6 The interaction between cytoplasmic protein and the PA1 probe is sensitive to modification of SH groups

Gel-retardation assay of binding reactions with 10 μ g of untreated 3T3 cytoplasmic extracts (lane 2) or with extracts treated either with alkaline phosphatase (lane 3) or with NEM (lane 4). Lane 1 contains RNAase T-digested probe alone. Note that a longer exposure than in previous Figures is presented. Control experiments for phosphatase and NEM treatments showed that AP-1 binding activity in nuclear extracts was modified in the expected manner (results not shown). Material migrating along with the largest RNAase T fragment of the probe most probably represents proteinase-digested fragments of the intact complex.

Figure 7 U.v. cross-linking of the PA1 probe and cytoplasmic proteins

Binding reactions between the PA1 probe and 10 μ g of 3T3 cytoplasmic extract were performed as in the gel-retardation experiments and the reaction products were irradiated on ice with 254 nm u.v. light, digested with a combination of RNAses A and T, and analysed by SDS/PAGE and autoradiography. Lane 1, probe alone; lane 2, probe plus 3T3 cytoplasmic extract; lane 3, 3T3 cytoplasmic extract treated with NEM before addition of probe. The mobilities of 14 C-labelled molecular mass standards are indicated on the left. The formation of the cross-linked complex was also inhibited by T1' oligonucleotide annealed to probe before binding (results not shown).

that was labelled in the cross-linking experiments (Figure 7, lane 2). Faint complexes of lower molecular size were seen in longer exposures. Incubation of the protein extract with NEM prior to binding and cross-linking inhibited the interaction (Figure 7, lane 3). Furthermore, when oligonucleotide T1' was allowed to anneal to the PA1 probe the formation of the 67 kDa complex

Table 2 Effect of dexamethasone on mRNA steady-state levels in transient transfections with COL1A1-hGH minigenes

Transient transfections were performed as described (Bornstein and McKay, 1988). RNA levels were determined by liquid hybridization and the values were corrected according to β -galactosidase expression from a co-transfected pRSV β -Gal plasmid. The combined results of three independent transfections are presented, and are means $\pm$ S.D. Control values without dexamethasone treatment are adjusted to 1.00.

Plasmid	Relative RNA levels		Decrease (%)
	Control	Dexamethasone	
pEhGH	1.00 $\pm$ 0.03	0.57 $\pm$ 0.21	33
pEhGH 2.2	1.00 $\pm$ 0.13	0.27 $\pm$ 0.23	73

Figure 8 Dexamethasone treatment inhibits the PA1-binding activity

(a) Gel-retardation assay of the PA1 probe and 3T3 cell extracts. Lanes 1 and 2, control extracts; lanes 3 and 4, extracts from cells treated for 24 h with 1 μ M dexamethasone. Cell extracts were prepared by rapid freezing-thawing cycles. The arrow indicates the position of the RNA-protein complex also obtained with cytoplasmic extracts (corresponding to α 1-RBF₆₇). Faster-migrating bands represent a smaller complex present in the freeze-thaw extracts and an RNAase T digestion product. (b) U.v. cross-linking of the PA1 probe and 10 μ g of cytoplasmic extract from human skin control fibroblasts (lane 1) or from fibroblasts treated with 1 μ M dexamethasone for 48 h prior to protein extraction (lane 2). Both lanes are from the same gel. Note that in this panel a longer exposure than for the cross-links in Figure 7 is presented. (c) U.v. cross-linking of the PA1 probe and 10 μ g of freeze-thaw extract from human fibroblasts. Lane 1, control; lane 2, cells treated with 1 μ M dexamethasone. In freeze-thaw extracts, a prominent 35 kDa complex is seen in addition to the α 1-RBF₆₇ complex.

was inhibited (results not shown), as in the gel-retardation experiments. Human skin fibroblast extracts produced a similar pattern, with the 67 kDa complex predominant (results not shown). We conclude that 3T3 cells and fibroblasts contain a specific cytoplasmic 67 kDa protein that interacts with the first highly conserved region of the α 1(I) collagen mRNA 3'-UTR. We call this protein α 1-RBF₆₇ (collagen α 1 chain RNA-binding factor of approx. molecular mass 67 kDa).

In subsequent experiments we studied the possible involvement of α 1-RBF₆₇ in the stability of α 1(I) collagen mRNA. In an ongoing study we have constructed a series of plasmids containing the putative regulatory domains of the COL1A1 gene and the hGH minigene as a reporter. In the present study we used two of these plasmids, pEhGH and pEhGH-2.2, to investigate the sequences needed for the down-regulation of collagen synthesis by corticosteroids. In pEhGH-2.2, the full-length 3'-UTR of

COL1A1 replaces the 3'-UTR of the hGH gene (Figure 1b). These plasmids were transiently transfected into 3T3 cells and the cells were chased for 48 h after transfection in either the presence or the absence of 1 μ M dexamethasone. Results from these experiments are summarized in Table 2. Dexamethasone treatment decreased the steady-state RNA level transcribed from plasmid pEhGH by about 33 %, whereas the RNA production from plasmid pEhGH-2.2 was reduced by 73 %. This suggests that the α 1(I) collagen promoter (in both plasmids) and the 3'-UTR (in pEhGH-2.2) both participate in the down-regulation of collagen expression by corticosteroids. This supports the results of Weiner et al. (1987) who found both transcriptional and post-transcriptional effects of dexamethasone on levels of α 2(I) collagen mRNA. To relate these results to the role of the α 1-RBF₆₇-RNA complex, we prepared freeze/thaw extracts from fibroblasts treated with dexamethasone for 24 or 48 h prior to harvesting of proteins. The freeze/thaw extracts were used in addition to cytoplasmic extracts for two reasons. First, a single 100 mm-diameter cell culture dish contains a sufficient number of cells for preparation of a freeze/thaw extract, which increases the number of replicate extracts in the experiments. Secondly, freeze/thaw extracts also include nuclear proteins. Thus one can evaluate whether the treatments used have a specific effect on cytoplasmic RNA-binding proteins. Because the specificity of α 1-RBF₆₇ was established in cytoplasmic extracts (Figures 3-5), we confirmed that binding of the 67 kDa protein in the freeze/thaw extracts was inhibited by T1' oligonucleotide, as in the cytoplasmic extracts (results not shown). Dexamethasone treatment had a specific effect on the protein-RNA interaction. The results of a gel-retardation experiment with 3T3 cell extracts are shown in Figure 8(a). Dexamethasone treatment for 24 h decreased α 1-RBF₆₇ activity significantly (Figure 8a, lanes 3 and 4) compared with that in control extracts (Figure 8a, lanes 1 and 2). This finding was reproduced in several label-transfer and gel-shift experiments using both 3T3 cells and human skin fibroblast extracts. In u.v. cross-linking experiments using cytoplasmic extracts of human skin fibroblasts the transfer of label to the α 1-RBF₆₇ protein was almost totally abolished in extracts from dexamethasone-treated cells (Figure 8b). Freeze/thaw extracts of human skin or tendon fibroblasts produced two distinct complexes (Figure 8c, lane 1) in u.v. cross-linking analyses, one of which had the same size as that produced by the cytoplasmic extracts. Further experiments indicated that the other complex (about 35 kDa) is putatively found mainly in nucleus (results now shown). When cell extracts were prepared from dexamethasone-treated cells, α 1-RBF₆₇ was again absent (Figure 8c, lane 2). It should be noted that dexamethasone did not decrease all RNA binding by fibroblast proteins. The amount of the smaller complex obtained from the freeze/thaw extracts (Figures 8a and 8c) remained at the same level as in the control extracts.

DISCUSSION

In this work we have characterized the binding of fibroblast proteins to a conserved domain of the 3'-UTR of human pro α 1(I) collagen mRNA transcribed *in vitro*. Cytoplasmic extracts were prepared from human skin fibroblasts and NIH 3T3 cells, which both produce type I collagen. The specificity of protein-RNA complex formation was evaluated by using competition assays with unlabelled RNA probes and oligodeoxyribonucleotides.

On the basis of the present results, we propose that a cytoplasmic factor, called α 1-RBF₆₇, recognizes and binds a conserved domain in the 3'-UTR of α 1(I) collagen mRNA. Inhibition of binding was observed when the extract was incubated with full-length unlabelled sense strand RNA probe but

not with the 5'-half of the sense strand RNA probe or with non-specific RNA (Figures 3 and 4). Several antisense and sense DNA oligonucleotides were tested in gel-shift experiments, and only the antisense oligonucleotide T1' inhibited the protein-RNA interaction (Figure 5). A similar approach utilizing DNA oligonucleotides to disturb the protein-RNA interaction has been successfully used in the analysis of testis-specific protein binding to the 3'-UTR of protamine-2 mRNA (Kwon and Hecht, 1991).

The computer-based model of the secondary structure of the PA1 probe (Figure 2) includes three distinct stems and loops, long stretches of possible single-stranded regions and several bulges in the stems. Bulges have recently been suggested to be the recognition targets for RNA-binding proteins (Roy et al., 1990; Calnan et al., 1991). The region covered by oligonucleotide T1', which inhibited α 1-RBF₆₇ binding to the PA1 probe, includes one such bulge structure and some single-stranded RNA. An approach using site-directed mutagenesis is needed, however, for the determination of the nucleotides at the exact binding site.

The u.v. cross-linking/label transfer method revealed that the α 1-RBF₆₇ protein of 3T3 cells and skin fibroblasts migrates as a single 67 kDa band in SDS/PAGE before and after reduction with 2-mercaptoethanol, which suggests that it consists of a single polypeptide chain (results not shown). The formation of cross-linked complex was sensitive to NEM treatment and the binding was inhibited by annealing of T1' oligonucleotide to the RNA prior to the binding reaction.

Several cytoplasmic proteins are now known to interact with the 3'-UTRs of different genes. One of the best examples is the 90 kDa iron-responsive-element-binding protein that has been cloned (Rouault et al., 1990). A distinct family of proteins seems to recognize the AUUU(A) element which is associated with the short half-life of several mRNAs (Shaw and Kamen, 1986). So far, proteins of 37 and 40 kDa in K562 cells (Brewer, 1991), a 32 kDa protein in HeLa nuclei (Vakalopoulou et al., 1991), inducible 30 and 43 kDa factors in T-cells (Bohjanen et al., 1991), and 28 and 45 kDa complexes (Malter, 1989) have been identified as AUUUA (or AU-rich element)-binding proteins. It should be noted that the part of the 3'-UTR used in this study contains no AUUUA motifs, and thus α 1-RBF₆₇ is most probably not related to the ARE-binding proteins. ARE-binding proteins recognizing *c-fos* mRNA also include a 66 kDa constitutive factor. This protein, however, binds to U-rich sequences (You et al., 1992), which are not present at the T1 site. A slightly larger protein of about 75 kDa is recognized by the *c-myc* stability determinant in the coding region of the mRNA (Bernstein et al., 1992). Interestingly, the greatest similarity between this 182 bp long *c-myc* stability determinant and the PA1 sequence maps to the T1 sequence (results not shown), but a possible relationship between α 1-RBF₆₇ and the 75 kDa protein remains open. Other known cytoplasmic mRNA-binding proteins include the 34 and 60 kDa liver proteins cross-linked to the 3'-UTR of apolipoprotein II mRNA (Ratnasabapathy et al., 1990). Given the inaccuracy of the label-transfer SDS/PAGE into account, we cannot fully exclude the possibility that α 1-RBF₆₇ is actually the same as some of the other described RNA-binding proteins within the 60–75 kDa molecular mass range.

Incubation of the cytoplasmic extract with NEM, a thiol group modifying agent, resulted in loss of binding (Figure 6). A similar inhibition by modification or oxidation of SH groups (presumably free cysteine residues) has been shown to inactivate the iron-responsive-element-binding protein (Hentze et al., 1989) and the AU-rich-element-binding protein (Malter and Hong, 1991). Free SH groups might thus be commonly used in the regulation of RNA-binding proteins. Furthermore, redox changes have been shown to regulate the activity of several

nuclear transcription factors, including *c-fos* (Abate et al., 1990). A nuclear factor, Ref-1, responsible for the reduction of SH groups, has been cloned from HeLa cells (Xanthoudakis et al., 1992). A common regulatory mechanism for the transcription factors and RNA-binding proteins might be used to produce concordant changes in both RNA synthesis and stability. It remains to be determined whether the α 1-RBF₆₇ protein also binds RNA in the nucleus during the processing of transcripts.

Alkaline phosphatase treatment of the cytoplasmic protein extracts did not alter α 1-RBF₆₇ binding (Figure 6, lane 3), suggesting that a phosphorylated form of the protein is not needed for the interaction. Phosphorylation has, however, been implicated in the activation of some other RNA-binding proteins, such as the AU-rich-element-binding protein (Malter and Hong, 1991) and those present in *Xenopus* oocytes (Murray et al., 1991).

The activity of α 1-RBF₆₇ seems to be sensitive to glucocorticoid treatment of the cells (Figure 8). Whether this is related to the observed decreased stability of α 1(I) mRNA in cortisol-treated skin fibroblasts (Hämäläinen et al., 1985) remains to be seen. This hypothesis was supported, however, by a pronounced decrease in the RNA steady-state level from a transiently transfected COL1A1 3'-UTR plasmid after dexamethasone treatment (Table 2). In the presence of dexamethasone the mRNA levels from plasmid pEhGH-2.2 decreased to about 30 % of the control value. This agrees with the observed decreases in α 1(I) and α 2(I) collagen mRNA levels to 30–50 % of control values after glucocorticoid exposure (Hämäläinen et al., 1985; Weiner et al., 1987). Moderately decreased RNA levels were observed from a plasmid carrying the COL1A1 promoter and the hGH 3'-UTR. Thus it seems that a plasmid carrying both the promoter and the 3'-UTR of the COL1A1 gene responds like the intact endogenous gene to corticosteroid hormones and probably contains all the regulatory elements needed. These results support the findings that both the transcriptional and post-transcriptional processes are involved in the down-regulation of the α 2(I) collagen gene in liver cells (Weiner et al., 1987). Glucocorticoids are known to destabilize several mRNAs, such as that of insulin (Philippe and Missotten, 1990), and to stabilize others, e.g. that of phosphoenolpyruvate carboxykinase (Petersen et al., 1991). The effect on the insulin mRNA can be inhibited by RU 486, a competitive inhibitor of dexamethasone binding to the glucocorticoid receptor. This suggests that the initial steps in transcriptional and post-transcriptional regulation by steroid hormones are similar or identical (Philippe and Missotten, 1990).

Changes in the stability of α 1(I) collagen mRNA can be produced by external stimuli, such as exposure of cells to hormones or cytokines. Transcriptional regulation of the gene in major collagen-producing tissues seems to be mediated by 5'-flanking sequence elements (Slack et al., 1991). During normal development and maintenance of connective tissues the COL1A1 transcript seems to be relatively stable. After cessation of growth, stabilization or destabilization of the α 1(I) mRNA is likely to be a major regulator in processes such as wound healing or inflammation which depend on local and rapid changes in the production of the extracellular matrix molecules.

The skilful technical assistance of Mrs. Marita Potila and Mrs. Liisa Peltonen is greatly appreciated. We thank Dr. Petri Auvinen for the critical reading and discussion of the manuscript and Dr. Pekka Mali for technical advice and discussions during the work. This study was financially supported by The Turku University Foundation, the Finnish Cultural Foundation (A.M.) and the Research and Science Foundation of Farnos Ltd. (A.M.).

REFERENCES

- Abate, C., Patel, L., Rauscher, F. J., III and Curran, T. (1990) *Science* **249**, 1157–1161

- Auwerx, J. and Sassone-Corsi, P. (1991) *Cell* **64**, 983–993
- Barsh, G. S., Roush, C. L. and Gelinas, R. E. (1984) *J. Biol. Chem.* **259**, 14906–14913
- Bernstein, P. L., Herrick, D. J., Prokipcak, R. D. and Ross, J. (1992) *Genes Dev.* **6**, 642–654
- Bohjanen, P. R., Petryniak, B., June, C. H., Thompson, C. B. and Lindsten, T. (1991) *Mol. Cell. Biol.* **11**, 3288–3295
- Bornstein, P. and McKay, J. (1988) *J. Biol. Chem.* **263**, 1603–1606
- Bornstein, P. and Sage, H. (1989) *Prog. Nucleic Acids Res.* **37**, 67–106
- Bouvet, P., Paris, J., Philippe, M. and Osborne, H. B. (1991) *Mol. Cell. Biol.* **11**, 3115–3124
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brewer, G. (1991) *Mol. Cell. Biol.* **11**, 2460–2466
- Calnan, B. J., Tidor, B., Biancalana, S., Hudson, D. and Frankel, A. D. (1991) *Science* **252**, 1167–1171
- Dhawan, J., Lichtler, A. C., Rowe, D. W. and Farmer, S. R. (1991) *J. Biol. Chem.* **266**, 8470–8475
- Hämäläinen, L., Oikarinen, J. and Kivirikko, K. I. (1985) *J. Biol. Chem.* **260**, 720–725
- Hentze, M. W., Rouault, T. A., Harford, J. B. and Klausner, R. D. (1989) *Science* **244**, 357–359
- Herget, T., Burba, M., Schmoll, M., Zimmermann, K. and Starzinski-Powitz, A. (1989) *Mol. Cell. Biol.* **9**, 2828–2836
- Iwai, Y., Bickel, M., Pluznik, D. H. and Cohen, R. B. (1991) *J. Biol. Chem.* **266**, 17959–17965
- Kähäri, V.-M., Chen, Y. Q., Su, M. W., Ramirez, F. and Uitto, J. (1990) *J. Clin. Invest.* **86**, 1489–1495
- Kwon, Y. K. and Hecht, N. B. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3584–3588
- Lee, K. A. W., Bindereif, A. and Green, M. R. (1988) *Gene Anal. Tech.* **5**, 22–31
- Määttä, A., Bornstein, P. and Penttinen, R. P. K. (1991) *FEBS Lett.* **279**, 9–13
- Malter, J. S. (1989) *Science* **246**, 664–666
- Malter, J. S. and Hong, Y. (1991) *J. Biol. Chem.* **266**, 3167–3171
- Moore, C. L., Chen, J. and Whoriskey, J. (1988) *EMBO J.* **7**, 3159–3169
- Mooslehner, K. and Harbers, K. (1988) *Nucleic Acids Res.* **16**, 773
- Murray, M. T., Krohne, G. and Franke, W. W. (1991) *J. Cell Biol.* **112**, 1–11
- Penttinen, R. P., Kobayashi, S. and Bornstein, P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1105–1108
- Petersen, D. D., Koch, S. R. and Granner, D. K. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7800–7804
- Petersen, R. B. and Lindquist, S. (1989) *Cell Regul.* **1**, 135–149
- Philippe, J. and Missotten, M. (1990) *Endocrinology (Baltimore)* **127**, 1640–1645
- Ratnasabapathy, R., Hwang, S.-P. L. and Williams, D. L. (1990) *J. Biol. Chem.* **265**, 14050–14055
- Rouault, T. A., Tang, C. K., Kaptain, S., Burgess, W. H., Haile, D. J., Samaniego, F., MacBride, O. W., Harford, J. B. and Klausner, R. D. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7958–7962
- Roy, S., Parkin, N. T., Rosen, C., Itovitch, J. and Sonenberg, N. (1990) *J. Virol.* **64**, 1402–1406
- Shapiro, D. J., Sharp, P. A., Wahli, W. W. and Keller, M. J. (1988) *DNA* **7**, 47–55
- Shaw, G. and Kamen, R. (1986) *Cell* **46**, 659–667
- Slack, J. L., Liska, D. J. and Bornstein, P. (1991) *Mol. Cell. Biol.* **11**, 2066–2074
- Smith, D. R., Jackson, I. J. and Brown, D. D. (1984) *Cell* **37**, 645–652
- Theil, E. C. (1990) *J. Biol. Chem.* **265**, 4771–4774
- Vakalopoulou, E., Schaack, J. and Shenk, T. (1991) *Mol. Cell. Biol.* **11**, 3355–3364
- Weiner, F. R., Czaja, M. J., Jefferson, D. M., Giambrone, M. A., Tur-Kaspa, R., Reid, L. M. and Zern, M. A. (1987) *J. Biol. Chem.* **262**, 6955–6958
- Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E. and Curran, T. (1992) *EMBO J.* **11**, 3323–3335
- You, Y., Chen, C.-Y. A. and Shyu, A.-B. (1992) *Mol. Cell. Biol.* **12**, 2931–2940
- Zuker, M. and Stiegler, P. (1981) *Nucleic Acids Res.* **9**, 133–148