

Nuclear and cytoplasmic $\alpha 1(I)$ collagen mRNA-binding proteins

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Abstract

We have recently identified a cytoplasmic protein, $\alpha 1$ -RBF₆₇, that specifically interacts with the conserved 3'-untranslated region of the $\alpha 1(I)$ collagen gene. The binding activity was decreased in extracts from dexamethasone treated cells, which correlates with the known accelerated turnover of the COL1A1 RNA [Määttä, A. and Penttinen, R.P.K. (1993) *Biochem. J.* 295, 691–698]. Now we report that a very similar protein is present in nuclear extracts of NIH 3T3, human fibroblast and HeLa cells, which suggests that determination of cytoplasmic mRNA stability is not the sole function of the $\alpha 1$ -RBF₆₇ activity. The binding to the RNA probe can be inhibited by annealing a DNA oligonucleotide or using excess of cold specific competitors. In UV-cross linking assays the nuclear protein has the same molecular weight (67 kDa) as the cytoplasmic one and the RNA-bound peptides generated by CNBr or V8 protease cleavage from both the cytoplasmic and the nuclear protein were identical. This protein was the only one of several nuclear collagen mRNA 3'-UTR binding proteins that was present in both nuclear and cytoplasmic extracts. In fibroblasts heparin-resistant nuclear RNA binding proteins had molecular weights of 45, 67 ($\alpha 1$ -RBF₆₇), and 71 kDa. HeLa-cells contained an additional protein of 51 kDa and several non-specific RNA-binding proteins. The binding activity is modified by changes in the redox state, which implicates that in the nucleus the binding affinities of $\alpha 1(I)$ collagen RNA-binding protein and AP-1, a redox sensitive nuclear factor, that is important in the transcription of $\alpha 1(I)$ collagen gene, can be regulated simultaneously to the same direction.

Key words: RNA-binding proteins; mRNA stability; Collagen

1. Introduction

In addition to transcriptional regulation, the level of collagen synthesis is determined by changes in the mRNA half-life. The stability of $\alpha 1(I)$ collagen mRNA can be modified by treatment of cells with different cytokines or hormones, alteration in cell adhesion and malignant transformation [1–3], but the regulatory mechanisms are poorly understood. In general, mRNA stability is controlled by an interplay of *cis*-acting RNA structures and *trans*-acting cellular proteins that either protect the mRNA or facilitate its degradation. Nuclear events in RNA metabolism include splicing of intron sequences and polyadenylation at the 3'-end of the maturing mRNA. After transport to cytoplasm the mRNA is subjected to the control of its half-life as a protein-mRNA complex. Some of the putative mRNA stability determining factors have been assigned to polyribosome particles and others have been identified in soluble cytoplasmic extracts [4,5]. Recently, these factors have also been found in the nucleus [6,7], suggesting that the same proteins might bind and protect nascent or newly proc-

essed nuclear RNAs and cytoplasmic mRNA until its degradation.

The 3'-UTR of the $\alpha 1(I)$ collagen mRNA contains two highly conserved domains around the alternate polyadenylation sites [8]. We have characterised a cytoplasmic RNA-binding protein, which we named $\alpha 1$ -RBF₆₇. This factor was shown to specifically interact with a short segment of the first conserved domain in the $\alpha 1(I)$ collagen mRNA [9]. The binding activity was markedly decreased in dexamethasone treated fibroblasts, which might be related to the well known finding that glucocorticoid hormones destabilize $\alpha 1(I)$ collagen mRNA. Moreover, transient transfection assays suggest that the 3-UTR is, indeed, needed for the decrease in $\alpha 1(I)$ collagen mRNA levels after dexamethasone treatment [9]. Thus, the $\alpha 1$ -RBF₆₇ binding activity seems to correlate with $\alpha 1(I)$ collagen mRNA stability.

The $\alpha 1(I)$ collagen gene has 51 short exons which indicates that the processing of the hnRNA to the corresponding mRNA is a complex process, during which the RNA should be protected against nucleases. This prompted us to study the nuclear proteins binding to the conserved parts of the $\alpha 1(I)$ collagen mRNA in order to find, whether $\alpha 1$ -RBF₆₇ or some other proteins are involved in the stabilisation of the mRNA in nucleus and during its transport to cytoplasm. The suggestions, that $\alpha 1(I)$ collagen mRNA can, on some occasions, be degraded already in the nucleus [10], further addressed the

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Abbreviations: AUBF, AU-rich element binding protein; $\alpha 1$ -RBF₆₇, $\alpha 1(I)$ collagen mRNA binding protein of 67 kDa; NEM, *N*-ethylmaleimide; TGF β , transforming growth factor β ; UTR, untranslated region.

importance of this issue. In this paper, we describe that a RNA binding activity similar to $\alpha 1$ -RBF₆₇ is also present in nuclear extracts along with several other RNA-binding proteins, that are not present in the cytoplasm.

2. Materials and methods

2.1. Cell lines and protein extracts

Cytoplasmic and nuclear extracts from 3T3 cells, human fibroblasts and suspension cultured HeLa cells were prepared using three different methods [11,12,13]. The cells were first incubated on ice for 10–15 min in a hypotonic buffer, and then disrupted by either dounce homogenisation [11,12] or passages through a 25-gauge hypodermic needle [13]. The nuclei were then pelleted by centrifugation for 30 s at 10,000 rpm [11], 15 min at 4000 rpm [12] or 30 s in a tabletop microfuge (14,000 rpm) [13] at +4°C. The supernatant was further cleared by re-centrifugation for 20 min at +4°C in a microfuge either as such [13] or after addition of KCl to 140 mM [12] and labelled as a cytoplasmic extract. The lysis of the cells and intactness of the nuclei was controlled by light microscopy. The nuclear pellets were briefly washed with a low salt buffer (0.02 M KCl) and extracted in 0.42 M [11,13] or 1.2 M KCl [12]. The extracts were stored in small aliquots in –70°C. In addition, RNA-binding activities were tested in cell extracts obtained by a 10 min lysis by 0.1% Triton X-100 on ice and pelleting of the nuclei and the cytoskeleton for 10 min at +4°C in a microfuge.

2.2. Production of labelled RNA-probes

Preparation of templates for in vitro RNA synthesis (Fig. 1) have been described [9], except the 0.15 *Ssp*I-*Hind*III fragment of the COL1A1 3'-UTR, which was subcloned from pGEM 0.3 *Taq*I-*Hind*III fragment into pBluescript KS (Stratagene). The probes were generated using the procedure recommended by the supplier for T7 and Sp6 RNA polymerases (Pharmacia).

2.3. RNA-protein binding assays

Gel retardation assays were performed as described [9]. In short, 2–5 × 10⁴ cpm of each probe was incubated with the protein extract at room temperature in the presence of 200 ng/μl *E. coli* tRNA and 2 mg/ml heparin. The reactions were digested with 100 U RNase T and the bound probe was resolved from free digestion products with a native 4.5% PAGE at +4°C. In experiments designed for the mapping of the binding site in the RNA, DNA oligonucleotides were annealed to the probe before the binding reaction by denaturing the probe and oligonucleotide for 5 min at +80°C and allowing the

mixture to renature to room temperature. The antisense T1' oligonucleotide (5'-TGGTAAGGTTGAATGCACTTTTG) maps 23 nucleotides upstream from the first polyadenylation signal. The other antisense oligonucleotide T2' (5'-GGTCAAAGATAAACTAAGTTTGAG) maps 66 nucleotides upstream from the same polyadenylation signal. UV-cross-linking assays were performed as described [7,9] except that heparin was omitted in some experiments. The cross-linked proteins were analysed by SDS-PAGE along with ¹⁴C-labelled molecular weight standards (Amersham) and visualised by autoradiography of dried gels. For the peptide mapping the cross-linked proteins were subjected for either 100 μg/μl V8 protease (Endoproteinase Glu-C, Boehringer Mannheim) for 3 h at +37°C or for 5 mg CNBr (Fluka, dissolved at 50 mg/ml in 70% formic acid) for 2 h at +22°C. CNBr cleaves the proteins at methionine residues and V8 protease (in the conditions used) at glutamic and aspartic acid residues. The digested samples were analysed by 12% SDS-PAGE in non-reducing conditions and autoradiography.

3. Results and discussion

The need to study the nuclear RNA-binding proteins that recognise the first conserved domain of the $\alpha 1$ (I) collagen 3'-UTR was prompted by the following reasons. Firstly, we suspected that the $\alpha 1$ (I) collagen mRNA might bind to protective proteins already in nucleus. Herget et al. [14] had also found in myoblasts a nuclear, single-stranded DNA binding protein that bound to approximately the same sequence as recognised by the $\alpha 1$ -RBF₆₇ in RNA [9]. Secondly, we found that like several other RNA-binding proteins, the $\alpha 1$ -RBF₆₇ was regulated by changes in the redox-state of free SH-groups; the binding was abolished when protein extracts were chemically modified by *N*-ethylmaleimide that alkylates SH-groups [9]. This regulation is similar to that which controls transcription factor binding to the AP-1 site in many genes. We and others have shown the importance of an intronic AP-1 site for the transcription of the $\alpha 1$ (I) collagen gene [15,16]. Thus, redox regulation in the nu-

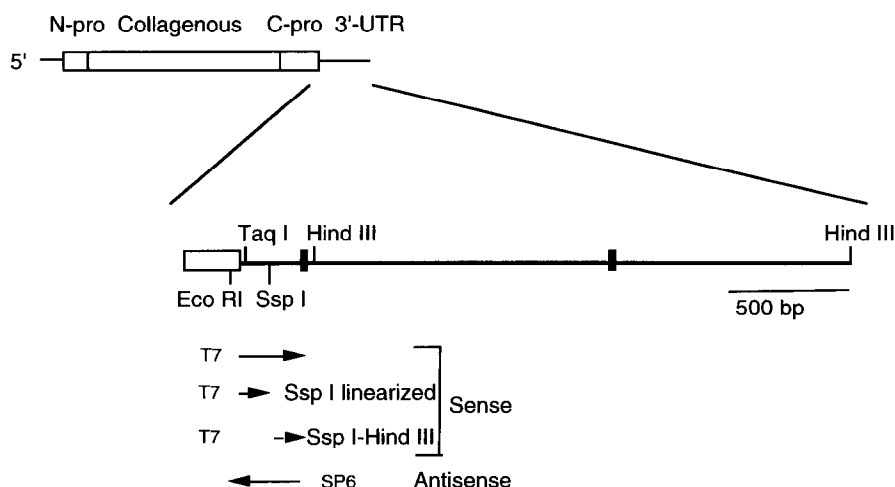


Fig. 1. Schematic representation of the human pro- $\alpha 1$ (I) collagen gene and its 3'-UTR. Open boxes denote coding sequences and black line 3'-UTR and flanking sequences. Black vertical bars represent the two alternate polyadenylation signals. The fragments subcloned into pGEM-1 (Promega) vectors and used for in vitro RNA synthesis are presented as arrows. These probes are referred in the subsequent Figures according to the restriction enzymes delineating them.

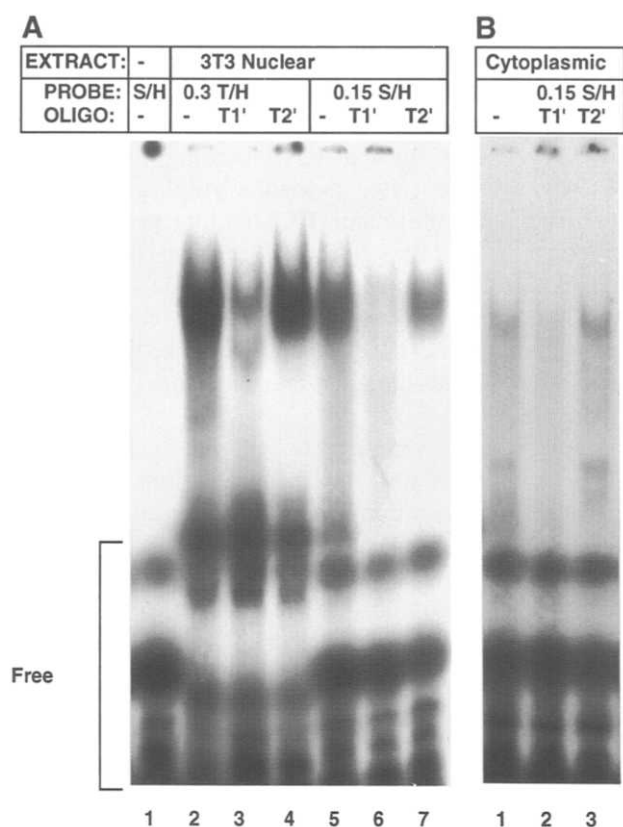


Fig. 2. Nuclear extracts contain RNA-binding protein binding to same site as $\alpha 1$ -RBF₆₇. (A) Formation of one complex is inhibited by T1' oligonucleotide annealed to the probe. Lane (1) RNase T digested 0.15 kb *SspI-HindIII* probe alone; (2) 0.3 kb *TaqI-HindIII* (PA1) probe without oligonucleotides bound to 10 μ g of 3T3 nuclear extract in the presence of *E. coli* tRNA and heparin, digested with RNase T; (3) PA1 probe annealed (see section 2) to T1' oligonucleotide mapping 20–40 nucleotides upstream from the polyadenylation site prior to the binding reaction; (4) PA1 probe annealed to T2' oligonucleotide prior to the binding reaction; (5) the 0.15 kb *SspI-HindIII* RNA probe bound to nuclear extract; (6) *SspI-HindIII* probe annealed to T1' oligonucleotide prior to the binding reaction; (7) *SspI-HindIII* probe annealed to T2' oligonucleotide prior to the binding reaction. In this experiment the extract was prepared as described in [13]. The gel was a 4.5% native polyacrylamide gel run in a Tris-glycine buffer at +4°C. (B) The oligonucleotide T1' inhibits RNA–cytoplasmic protein interaction. The 0.15 kb *SspI-HindIII* probe was denatured and allowed slowly to anneal with indicated oligonucleotides. Binding reactions were performed with 10 μ g of 3T3 cytoplasmic proteins. Lane (1) probe denatured and renatured without oligonucleotides; (2) probe annealed to oligonucleotide T1'; (3) probe annealed to oligonucleotide T2'.

cleus could have a simultaneous effect both on transcription factors of the $\alpha 1$ (I) collagen gene and on putative regulators of the half-life of its mRNA. Thirdly, some other nuclear RNA-binding proteins have been suggested to participate in the control of mRNA stability [6]. Furthermore, nuclear RNA-binding proteins are major candidates for the control over the usage of the two alternate polyadenylation sites. The resulting two mRNAs of 4.7 and 5.8 kb sizes have different stabilities and, e.g. respond in a different way to TGF- β application [17].

We used the *TaqI-HindIII* (PA1) fragment that covers the whole 3'-UTR of the 4.8 kb mRNA species and its 5' and its 3'-terminal halves restricted at the *SspI* site as templates for in vitro RNA probe synthesis (Fig. 1). Gel retardation assays were performed using nuclear and cytoplasmic extracts of 3T3 cells. Binding assays with nuclear proteins were carried out in the same assay buffer as with cytoplasmic extracts.

Gel retardation experiments with the 0.3 kb PA1 RNA probe and nuclear extract resulted in a broad smear migrating approximately at the position of $\alpha 1$ -RBF₆₇-PA1 RNA complex (Fig. 2A, lane 2). Previously we showed by competition assays with unlabeled RNA-transcripts and DNA oligonucleotides that the binding site of $\alpha 1$ -RBF₆₇ resides in the 3'-terminal end of the 0.3 kb PA1 probe, ca. 20–40 nucleotides upstream from the triplicate AATAAA polyadenylation signal [9]. When T1' oligonucleotide was annealed to the PA1 probe before the binding reaction, two retarded complexes were still seen, but some material of the original complexes was lost (Fig. 2A, lane 3). T2' oligonucleotide had no effect on the formation of the complex migrating like the $\alpha 1$ -RBF₆₇ activity (Fig. 2A, lane 4). The 3'-terminal fragment was also used as a template for the RNA-probe for binding experiments with nuclear and cytoplasmic extracts. A single complex was formed between the *SspI-HindIII* probe and nuclear extract (Fig. 2A, lane 5). The complex formation was prevented by annealing of the oligonucleotide T1' to the probe (Fig. 2A, lane 6), but not by annealing the oligonucleotide T2' (Fig. 2A lane 7). The results were similar when cytoplasmic extracts were used (Fig. 2B). When oligonucleotide T1' was annealed to the *SspI-HindIII* probe prior to the binding reaction no complex formation was seen (Fig. 2B, lane 2), whereas the T2' oligonucleotide annealing the near vicinity of the T1 site did not prevent the binding of cytoplasmic proteins (Fig. 2B, lane 3) confirming the results obtained with the longer 0.3 kb PA1 probe [9]. Finally, in competition assays with nuclear extracts and the 3' terminal *SspI-HindIII* probe, the specific unlabeled transcript perturbed the interaction between the probe and nuclear extracts (Fig. 3A; lane 4) like in the reactions with the cytoplasmic protein and RNA-probe [9]. We thus conclude, that both nuclear and cytoplasmic extracts contain a protein that binds to the same site in the $\alpha 1$ (I) collagen 3'-UTR RNA. Besides the $\alpha 1$ -RBF₆₇ which binds to the 3'-terminal *SspI-HindIII* fragment, the full-length 0.3 kb PA1 probe bound other nuclear proteins (compare Fig. 2A, lanes 2 and 3 to lanes 5 and 6) indicating that additional proteins might interact with the 5' terminal part of the PA1 probe.

The approximate sizes of the proteins participating in the complexes were determined by UV cross-linking. In this method the binding reactions are irradiated with UV-light, which results in covalent attachment of probe nucleotides to bound proteins. Label transferred to pro-

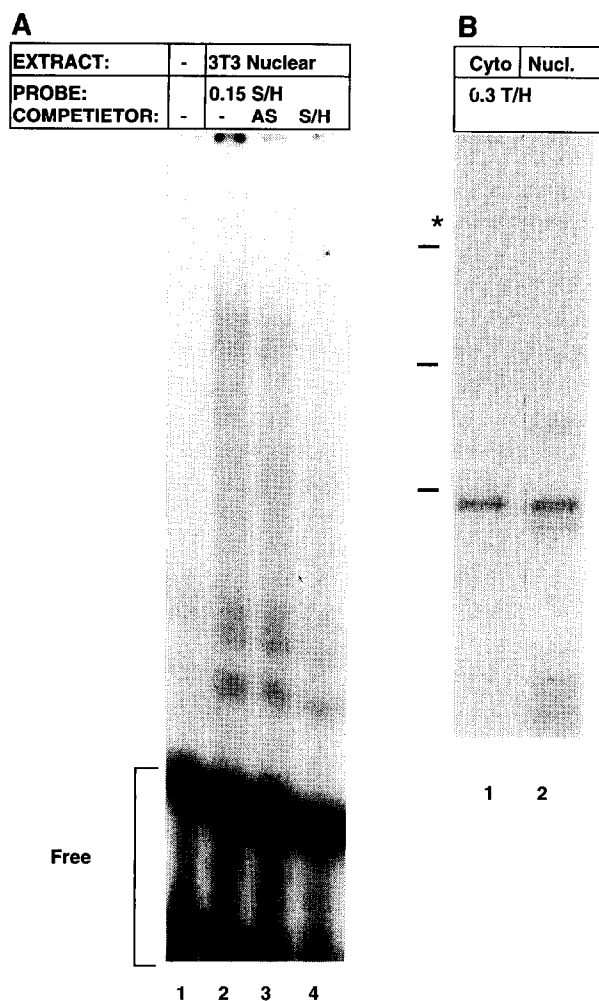


Fig. 3. (A) Competition experiment with the 3T3 nuclear extract and the 0.15 *SspI-HindIII* probe. Unlabeled RNA-probes (all in approximately 120 \times excess over the labelled probe) were allowed to bind nuclear proteins 10 min prior to addition of the labelled probe. Lane (1) probe alone; (2) no competitors; (3) excess of antisense (AS) RNA; (4) excess of unlabeled sense *SspI-HindIII* RNA. (B) UV cross-linking of PA1 probe and 3T3 extracts. Cross-linking of 10⁵ cpm of probe and 20 μ g of cytoplasmic (lane 1) or nuclear (lane 2) protein extract was carried out with 254 nm light at a distance of 2 cm for 15 min on ice in the presence of 2 mg/ml heparin and 200 ng/ μ l *E. coli* tRNA. RNase-digested reactions were run in non-reducing conditions in a 7.5% SDS-PAGE. The asterisk indicates the interface between the stacking and separating gels, and the bars indicate the migration of 200 kDa, 97 kDa and 69 kDa molecular weight standards.

teins visualises them in autoradiography after SDS-PAGE. If T1' oligonucleotide was annealed to the probe before binding and irradiation in these assays, neither the whole 0.3 kb PA1 probe nor the 0.15 kb *SspI-Hind* probe cross-linked to the cytoplasmic proteins (not shown). One major radioactive band was seen when cytoplasmic protein extract was used with both of these probes without the annealed oligonucleotide. This protein migrated as a single 67 kDa polypeptide in SDS-PAGE [9], (seen also in Fig. 3B, lane 1). Experiments with nuclear extract from 3T3 cells and the PA1 probe demonstrated several proteins that had acquired label from the RNA probe.

One of them had a 67 kDa mobility equal to α 1-RBF₆₇ in cytoplasmic extracts (Fig. 3B, lane 2). Similar results were obtained when human fibroblast nuclear and cytoplasmic extracts were used, although in human cells, the pattern of other nuclear RNA-binding proteins was somewhat different (see below). Cytoplasmic extracts contained only one major PA1-binding protein corresponding to α 1-RBF₆₇, although in some reactions we have also seen a complex of over 200 kDa size in fibroblasts (not shown). Thus, nuclear extracts contain a protein that migrates in the electrophoresis like the cytoplasmic α 1-RBF₆₇ and binds to the same short segment of PA1 probe.

Three methods were used to generate nuclear and cytoplasmic extracts and, furthermore, Triton X-100 lysis was used for preparation of a cytoplasmic extract. In one of the methods swollen cells are lysed with passages through a 25-gauge needle and in the two other methods by dounce homogenisation. The latter methods differ in the conditions used for the centrifugation of the nuclei. All the extracts contained α 1-RBF₆₇. None of the other nuclear α 1(I) collagen mRNA-binding proteins were found in cytoplasmic or Triton extracts. Previously, distribution of nuclear and cytoplasmic hnRNP proteins [18] and an AU-rich element binding protein [19] have been established by comparable extracts. We tested the extracts also for the distribution of the transcription factor Sp1. Binding of this protein to its double stranded consensus binding site oligonucleotide was seen when the nuclear extract was used but not when either the cytoplasmic extracts or the Triton X-100 extract was used (not shown).

It is most likely that α 1-RBF₆₇ is present both in the nucleus and cytoplasm, whereas most of the nuclear RNA-binding proteins that recognise the 3'-UTR of α 1(I) collagen mRNA are not detectable in cytoplasmic extracts. Additional evidence for the identity of the nuclear and cytoplasmic α 1-RBF₆₇ activities was obtained by peptide mapping. The proteins cross-linked to the *SspI-HindIII* probe were digested by either V8 protease or CNBr and the peptide bound to RNA probe was visualised by autoradiography after SDS-PAGE. V8 protease digestion of the protein-RNA complex resulted in a approximately 14 kDa peptide with both the cytoplasmic and nuclear extracts, and the CNBr cleavage resulted in a peptide of about 40 kDa (not shown).

These findings bear direct consequences to explaining roles of α 1-RBF₆₇. It is the major cytoplasmic protein that can be shown with the current methods to interact with the conserved 3'-UTR domain of α 1(I) collagen mRNA in fibroblasts. Furthermore, the binding activity is decreased when cells are treated with glucocorticoid hormones that decrease the stability of the α 1(I) collagen mRNA. The present results are also consistent with the hypothesis that α 1-RBF₆₇ binds to the already in the nucleus and is transported to the cytoplasm as the

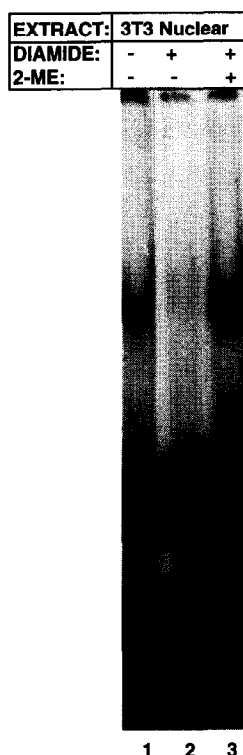


Fig. 4. Diamide treatment abolishes reversibly the RNA–protein binding activity. 3T3 nuclear extract was treated without (lane 1) or with (lanes 2 and 3) 5 mM diamide at room temperature, 2-mercaptoethanol (5% final) was added to reaction in lane 3 for additional 10 min. Binding reaction and electrophoresis were performed as described in Fig. 2.

mRNP complex. Further experiments are needed to dissect, whether $\alpha 1$ -RBF₆₇ participates mainly in the transport and targeting of mRNA or in the maintenance of its stability.

A few RNA-binding proteins are known to shuttle between nucleus and cytoplasm. Recently, a tRNA binding protein in HeLa nuclei was identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and it was suggested that it functions in the nuclear export of tRNA [20]. In a similar way, several hnRNA binding proteins have been shown to actively shuttle between nucleus and cytoplasm and to bind mRNA in the cytoplasm [18]. Proteins associated with RNA stability are also found in the nucleus. A 32 kDa polypeptide that recognises AU-rich domains of several short-lived mRNAs is found predominantly in the nucleus, although it is present in the cytoplasm, too [6]. Interestingly, one AU-rich element binding protein, AUBF has been shown to increase the export of AU-rich RNAs from nucleus to cytoplasm [7]. At the same time, AUBF putatively protects RNA from the nuclear matrix associated endoribonuclease [7]. Highly conserved 3' UTRs and their cognate binding proteins may, indeed, have several simultaneous and unrelated tasks. For example, all described cases of mRNA localisation and subsequent non-uniform distribution in cytoplasm are mediated by *cis*-acting 3'-UTR sequences (discussed in [21]).

We have also found that the binding mechanism of the cytoplasmic protein putatively involves free SH-groups in cysteine residues because *N*-ethylmaleimide treatment of the cytoplasmic extracts abolishes the binding. We wanted to verify, whether the redox change also regulates the binding of the nuclear counterpart of this binding activity. To that purpose we used diamide, an oxidising agent, the effect of which is, unlike that of NEM, reversible by reduction with 2-mercaptoethanol [22]. A 10-min incubation of the nuclear extract with 10 mM diamide effectively inhibited the subsequent complex formation of $\alpha 1$ -RBF₆₇ with the *Ssp*I-*Hind*III RNA (Fig. 4), and the effect of diamide was reverted by application of 2-mercaptoethanol for another 10 min after diamide but before addition of the probe (Fig. 4, lane 3).

Redox regulation of transcription factors and RNA-binding proteins is a relatively newly found mechanism [22,23]. Binding of nuclear factors to an intronic AP-1 site of $\alpha 1$ (I) collagen gene appears to be an important regulator of transcription [15,16]. We have previously shown that redox changes modulate the AP-1 binding at this site [16], and now present evidence for redox regulation of $\alpha 1$ (I) collagen RNA binding protein, $\alpha 1$ -RBF₆₇, in nucleus. Thus, potential regulators of transcription and mRNA transport/stability could be inactivated or activated simultaneously. This might be a part of the mechanism that causes the well-documented parallel changes both in transcription and in mRNA stability of $\alpha 1$ (I) collagen gene (e.g. [1,2,3,17]).

In the course of these studies we found that the amount and specificity of the nuclear RNA-binding proteins interacting with the PA1 probe varied in different cells. Many of these experiments were carried out with-

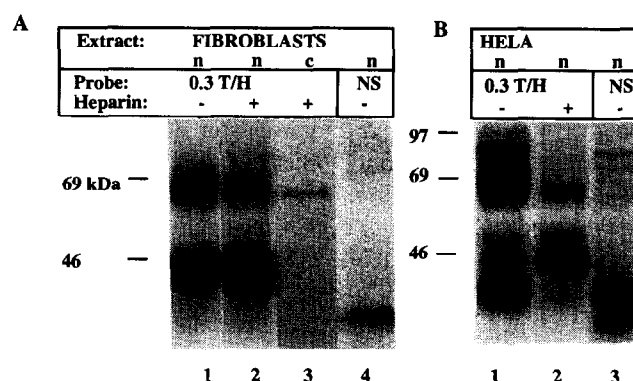


Fig. 5. Collagen RNA binding proteins are more specific in fibroblasts than in HeLa cells. (A) SDS-PAGE analysis of the RNA–protein complex formation in human fibroblasts. 10 μ g fibroblast protein extract [11] (lanes 1, 2 and 4 nuclear proteins; lane 3 cytoplasmic proteins, c) were cross-linked to 0.3 kb *Taq*I-*Hind*III PA1 sense probe or to a probe transcribed from a *Pvu*II digested pBluescript KS (Stratagene) plasmid (NS; lane 4) either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 1 mg/ml heparin. (B) UV cross-linking of HeLa nuclear extract proteins [11] to PA1 sense probe. Lane (1) heparin omitted; (2) 1 mg/ml heparin added; (3) pBluescript-derived non-specific probe.

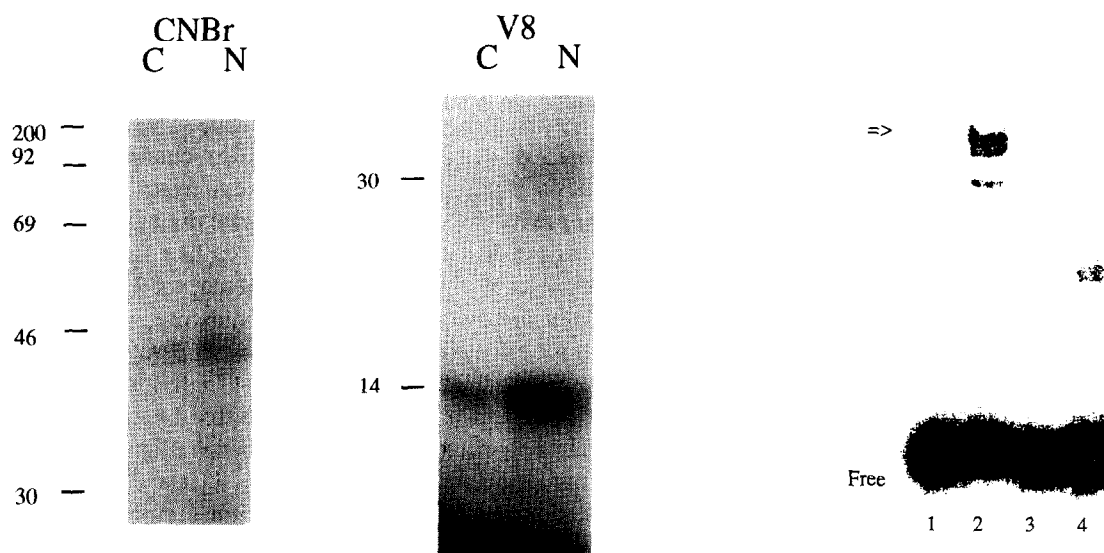


Fig. 6 (A) Sp1 consensus oligonucleotide 5'-GCTCCAAGGGGCGGGCAACCCAGGG was labelled with [γ - 32 P]dATP, annealed to its complementary strand and used in electrophoretic mobility shift assays. The binding reactions included 10 000 cpm of the probe 10 μ g of the extracts and 2 μ g of poly d[I.C]. Lane (1) probe alone, lane (2) nuclear extract, lane (3) cytoplasmic extract, lane (4) Triton extract. The arrow indicates the specific Sp1 complex (as evaluated by competition assays, mobility of the purified Sp1 protein and supershifts with specific antisera). The figure is a photocopy of an autoradiogram. (B) The nuclear and cytoplasmic extracts of 3T3 cells were UV cross-linked to the 0.15 *SspI-HindIII* probe and cleaved with CNBr or V8 protease as described in section 2. CNBr-digested samples were evaporated to dryness before electrophoresis. V8 digestions giving identical results were performed with extracts described in the references [12 and 13]. The CNBr cleavages were performed with the small-scale extracts [13] only.

out heparin to reveal also non-specific RNA–protein interactions. To characterise the additional proteins, we utilised HeLa cell nuclear extract and performed binding experiments with varying heparin concentrations and with different RNA-probes. As a control 'nonsense' probe, a *PvuII* digested pBluescript plasmid was used. Addition of heparin had only a minor effect on protein binding when fibroblast nuclear extract was used and complexes of 45, 67 (α 1-RBF₆₇) and 71 kDa were seen with or without heparin (Fig. 5A, lanes 1 and 2). HeLa nuclear extract contained, on the contrary, several proteins that reproducibly were recognised by the PA1 probe: three proteins of 34–36, 45 and 51 kDa molecular weights were accompanied by a cluster of proteins in a size range between 65 and 103 kDa (p65, p67, p71, p80, p87, p103) (Fig. 5B, lane 1.). Heparin treatment efficiently inhibited the binding of some of the proteins leaving a doublet at about 67 kDa and single prominent bands of 45 and 51 kDa (Fig. 5B lane 2). It should be noted that in 3T3 nuclear extract the pattern of the complexes (Fig. 3A) was somewhat different, apart α 1-RBF₆₇, (p65, p67, p80) from that in either human cell lines.

The use of the pBluescript control probe (NS) turned out to be very informative on the specificity of the nuclear RNA–protein binding. At least five proteins of HeLa nuclear extract bound to this probe (Fig. 5B, lane 3). These complexes had the same mobility as the heparin sensitive complexes of same extract probed with the PA1 sequences indicating that the 34–36, 80 and 87 kDa pro-

teins are non-specific RNA-binding proteins present in the HeLa but absent in the fibroblast nuclear extracts. The fibroblast extract contained detectable amounts of only the 32 kDa nonsense probe binding protein (Fig. 5A, lane 4), further indicating the specificity of the collagen RNA-binding proteins present in these cells. These results further address that there are several specific and non-specific proteins in the nuclear extracts that can bind the highly conserved 3'-UTR of α 1(I) collagen, and only the one corresponding to α 1RBF₆₇ is found also in cytoplasmic extracts. It might also be that a more differentiated phenotype expresses a select subset of RNA-binding proteins and the large amount of non-specific proteins present in the HeLa extract reflects the transformed state of the cell line. The 51 kDa protein was present only in the HeLa cells, but was not characterised in detail. This protein might be interesting in further experiments, because it has been demonstrated that in HeLa cells, pro α 1(I) collagen expression is strongly down-regulated at the post-transcriptional level [24].

In conclusion, the conserved 3'-UTR common to both α 1(I) collagen mRNA species binds several nuclear proteins. One of them appears to be similar and perhaps identical to a previously described cytoplasmic RNA-binding protein, α 1-RBF₆₇, which might thus participate also in mRNA transport or localisation. The enhancement of binding activity via reduction of cysteine residues further addresses α 1-RBF₆₇ as a candidate for post-transcriptional determinant of α 1(I) collagen gene expression.

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References

- [1] Bornstein, P. and Sage, H. (1989) *Prog. Nucleic Acids Res.* 37, 67–106.
- [2] Dhawan, J. and Farmer, S.R. (1990) *J. Biol. Chem.* 265, 9015–9021.
- [3] Slack, J.L., Parker, M.I., Robinson, V.R. and Bornstein, P. (1992) *Mol. Cell. Biol.* 12, 4714–4723.
- [4] Hentze, M.W. (1991) *Biochim. Biophys. Acta* 1090, 281–292.
- [5] Schiavi, S.C., Belasco, J.G. and Greenberg, M.E. (1992) *Biochim. Biophys. Acta Rev. Cancer* 1114, 95–106.
- [6] Vakalopoulou, E., Schaack, J. and Shenk, T. (1991) *Mol. Cell. Biol.* 11, 3355–3364.
- [7] Müller, W.E.G., Slor, H., Pfeifer, K., Hühn, P., Bek, A., Orsulic, A., Ushijima, H. and Schröder, H.C. (1992) *J. Mol. Biol.* 226, 721–733.
- [8] Määttä, A., Bornstein, P. and Penttinen, R.P.K. (1991) *FEBS Lett.* 279, 9–13.
- [9] Määttä, A. and Penttinen, R.P.K. (1993) *Biochem. J.* 295, 691–698.
- [10] Askew, G.R., Wang, S. and Lukens, L.W. (1991) *J. Biol. Chem.* 266, 16834–16841.
- [11] Shapiro, D.J., Sharp, P.A., Wahli, W.W. and Keller, M.J. (1988) *DNA* 7, 47–55.
- [12] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) (1993) *Current Protocols in Molecular Biology*, Suppl. 24, Wiley Interscience.
- [13] Lee, K.A.W., Bindereif, A. and Green, M.R. (1988) *Gene Anal. Techn.* 5, 22–31.
- [14] Herget, T., Burba, M., Schmoll, M., Zimmermann, K. and Starzinski-Powitz, A. (1989) *Mol. Cell. Biol.* 9, 2828–2836.
- [15] Liska, D.J., Slack, J.L. and Bornstein, P. (1990) *Cell Regul.* 1, 487–498.
- [16] Määttä, A., Glumoff, V., Paakkonen, P., Liska, D., Penttinen, R.P.K. and Elima, K. (1993) *Biochem. J.* 294, 365–371.
- [17] Penttinen, R.P., Kobayashi, S. and Bornstein, P. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1105–1108.
- [18] Pinol-Roma, S. and Dreyfuss, G. (1992) *Nature* 355, 730–732.
- [19] Zhang, W., Wagner, B.J., Ehrenman, K., Schaefer, A.W., DeMaria, C.T., Crater, D., DeHaven, K., Long, L. and Brewer, G. (1993) *Mol. Cell. Biol.* 13, 7652–7665.
- [20] Singh, R. and Green, M.R. (1993) *Science* 259, 365–368.
- [21] Duret, L., Dorkeld, F. and Gautier, C. (1993) *Nucleic Acids Res.* 21, 2315–2322.
- [22] Hentze, M.W., Rouault, T.A., Harford, J.B. and Klausner, R.D. (1989) *Science* 244, 357–359.
- [23] Abate, C., Patel, L., Rauscher III, F.J. and Curran, T. (1990) *Science* 249, 1157–1161.
- [24] Furth, J.J., Wroth, T.H. and Ackerman, S. (1991) *Exp. Cell Res.* 192, 118–121.