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Purification of a novel factor which binds to the mouse α2 (I) collagen promoter

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We have identified and purified a DNA binding protein which specifically binds to a segment of the mouse α2 (I) collagen promoter between −420 and −399 bp upstream of the start of transcription. Purification included heparin-agarose and sequence-specific DNA-affinity chromatography, followed by SDS-PAGE and renaturation of the DNA binding activity after elution from SDS-polyacrylamide gel. The DNA binding activity resides in two species of 42 kDa and 40 kDa, respectively. The levels of DNA binding activity of this factor, which has been tentatively designated as ColF1, are considerably higher in nuclear extracts of NIH-3T3 fibroblasts than in nuclear extracts from epidermal cells, lymphoid cells and transformed NIH-3T3 fibroblasts.

DNA binding factor; Mouse α2 (I) collagen promoter; NIH-3T3 fibroblast

1. INTRODUCTION

Type I collagen is a major protein of the extracellular matrix present at high concentrations in bones, tendons, skin, cornea and in much lesser concentrations in a number of other tissues. Changes in the biosynthesis of type I collagen occur under several physiological conditions such as during embryonic development of these organs and also during wound healing. Furthermore, such changes also occur in a number of pathological conditions involving fibrotic lesions of various organs. Although translational control [1] has been proposed to occur in some situations, transcriptional regulation of type I collagen genes plays a major role in the biosynthesis of this protein [2–6].

Several factors present in nuclear extracts of fibroblasts were previously shown to interact with specific DNA sequences in the α1 (I) and α2 (I) collagen promoters [7–12]. These include a heterodimeric CCAAT binding factor (CBF) which binds to a CCAAT motif which is located between −96 and −100 in the α1 (I) collagen promoter and between −80 and −84 bp in the α2 (I) promoter [7,8,10,12]. We have shown previously that CBF activates transcription of both the α1 (I) and α2 (I) collagen promoters in a reconstituted in vitro transcription system [10]. In addition, nuclear factor I or a factor with similar binding properties binds to a sequence between −315 and −295 in the α2 (I) collagen promoter [9]. Other *cis*-acting elements have been identified in these two genes that are binding sites for still other specific factors [11–15]. Based on mutation analysis of the DNA binding sites, some of these factors are believed to act as positive effectors of transcription whereas others appear to have an inhibitory role in transcription [10–12]. We report here on the isolation and characterization of an additional factor which binds to a segment between −419 and −399 bp in the α2 (I) collagen gene 5′ flanking sequence.

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2. MATERIALS AND METHODS

2.1. *DNA fragments and plasmids*

The *Xba*I–*Xma*I fragment contains the sequence between −504 and −105 of the mouse α2 (I) collagen promoter; the *Apa*I–*Hin*dIII fragment contains the sequence between −105 and +54 in this promoter [16]. The plasmid pAH5 was obtained by cloning two 31-mer complementary oligonucleotides corresponding to the sequence between −394 and −424 of the mouse α2 (I) collagen promoter into the *Sma*I site of plasmid pUC-12. A 72 bp *Hin*dIII–*Eco*RI fragment was prepared from this recombinant plasmid and also includes surrounding polylinker sequences. Labeled restriction fragments for the DNA binding assays were prepared by phosphorylation of the 5′ ends using T4 polynucleotide kinase and [γ-^{32}P]ATP.

2.2. *Preparation of nuclear extracts*

NIH-3T3 fibroblasts and A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. NIH-3T3 cells were transformed by either infection with the Moloney murine sarcoma leukemia virus complex (MMSV) or by transfection with a plasmid containing the v-*ras* oncogene. Mouse epidermal cells (HEL cells) [17] were grown in Dulbecco's modified Eagle's medium

with 10% fetal calf serum. A mouse T-lymphocyte cell line kindly supplied by Dr. Riethmüller, University of Munich was grown in RPMI supplemented with 10% FCS and 20 U/ml IL-2. Preparation of nuclear extracts for purification of the ColF1 binding factor was performed as described previously [7] from NIH-3T3 cells except that 1 μg/ml pepstatin A and 0.01% Nonidet P-40 were included in all buffers. For detection of binding activity and the exonuclease III assay 2–6 × 10^6 cells were harvested and washed twice with phosphate buffer saline at 4°C. The nuclei were isolated according to Groudine et al. [18]. The nuclei were then suspended in 4 vols. of 20 mM HEPES, pH 7.9, 1 mM EGTA, 0.5 mM DTT, 400 mM NaCl, 10% glycerol, 0.01% Nonidet P-40, 0.5 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A. The mixture was stirred for 30 min at 4°C and centrifuged for 10 min in an Eppendorf centrifuge. The supernatants were used as nuclear extracts. Protein concentrations were assayed with a Bio-Rad kit [19].

2.3. *Assay of DNA binding proteins*

The exonuclease III assay [20] has been described previously. For the gel retardation assay 1–3 μl of nuclear extracts were incubated for 20 min at 26°C in 10 μl of a mixture containing 20 mM HEPES, pH 7.5, 0.5 mg/ml bovine serum albumin, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 4 μl of poly(dI-dC) as well as 10^4 cpm (approximately 0.2 ng) of ^{32}P-labeled DNA fragments. To stop the binding reaction, 2.0 μl of a sample buffer containing 0.1% xylene cyanol, 0.1% Bromphenol blue and 20% glycerol was added, and the DNA–protein complexes were separated from other components of the mixture by electrophoresis on a 6% polyacrylamide gel in TBE (89 mM Tris, 89 mM boric acid, 8 mM EDTA pH 7.9). The binding activity was measured by densitometric scanning of the autoradiograph. In some experiments the regions of the gel containing the labelled species were cut out and counted by liquid scintillation.

2.4. *Purification of the binding factor from NIH-3T3 cells*

A nuclear extract (65 ml) prepared from 40 g (wet weight) of NIH-3T3 cells was dialyzed against buffer A containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol, 0.01% Nonidet P-40, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. The dialyzed extract was applied to a column (8 ml) of heparin-agarose. The column was washed with 40 ml of buffer A and then washed with 11 ml of the same buffer containing 0.35 M NaCl. The ColF1 binding activity was eluted with 0.75 M NaCl in buffer A. This fraction was purified by sequence-specific oligonucleotide affinity chromatography [21]. The affinity column consisted of a double-stranded oligonucleotide (corresponding to the sequence between −394 and −424) covalently linked through a 10-nucleotide long 5′ overhang of one DNA strand to CNBr-activated Sepharose-4B. The active fractions from the heparin-agarose column were pooled, diluted 15-fold with NaCl-free buffer A and applied to this DNA-affinity column (0.7 ml). The column was then washed with 100 ml of buffer A and 100 ml of buffer A containing 0.3 M NaCl. The ColF1 binding activity was eluted with 1.5 ml of the same buffer containing 1.0 M NaCl. The active fractions, diluted in 20 vols. of NaCl-free buffer A, were reapplied on another column containing the same DNA-affinity resin (0.7 ml) and washed with the following volumes of buffer A with the indicated concentration of NaCl: 50 mM, 100 ml; 150 mM, 100 ml; 350 mM, 30 ml; 500 mM, 1.5 ml; 0.75 mM, 1.5 ml; 1.0 M, 1.5 ml; 1.5 M, 1.5 ml. The fractions were analyzed by gel retardation assay also by SDS-polyacrylamide gel for silver-staining according to Wray et al. [22].

2.5. *Characterization of the binding factor by FPLC*

Fractions purified once by DNA affinity chromatography were applied to an FPLC MonoQ column (Pharmacia) equilibrated with 30 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM PMSF, 50 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. A 20 ml gradient of 50 to 500 mM NaCl in the same buffer was applied for elution.

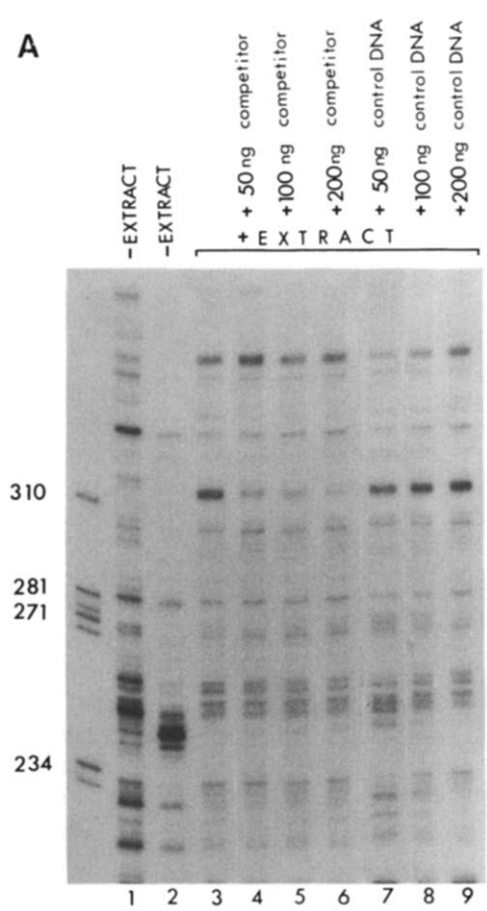
2.6. *Renaturation of the ColF1 factor*

400 μl (approximately 4 μg of protein) of the fraction purified once on the affinity column was run on an SDS-polyacrylamide gel. The protein bands were excised and eluted from the polyacrylamide gel. Renaturation was performed according to Briggs et al. [23]. The SDS was removed from the protein by acetone precipitation, and the precipitated protein was resuspended in 6 M guanidine hydrochloride (Gu-HCl) to denature the polypeptide chains. Subsequent removal of the Gu-HCl by dialysis allowed refolding of the polypeptide chains and recovery of DNA binding activity as determined by the gel retardation assay.

3. RESULTS AND DISCUSSION

In order to identify factors binding to the promoter region of the mouse α2 (I) collagen gene, a series of fragments spanning the sequences between −990 and +54 have been tested by either DNase I or exonuclease III footprinting using nuclear extracts isolated from mouse NIH-3T3 fibroblasts as a source of DNA binding proteins [8]. Using the exonuclease III assay, with an *Xma*I–*Xba*I fragment between −504 and −105 labeled at the *Xma*I site at −105, a DNA binding factor was identified that showed a 5′ boundary of protection mapping approximately at −420 (Fig. 1a). This boundary was confirmed more precisely by running the four Maxam–Giblert sequencing reactions of the same fragment in parallel to the exonuclease III reaction (data not shown). Specificity of binding was established by competition experiments which demonstrated that increasing amounts of an unlabeled fragment containing sequences of the α2 (I) collagen promoter between −419 and −399, decreased the exonuclease III generated signal in a dose-dependent manner, whereas the same amounts of an unrelated DNA fragment did not compete for binding (Fig. 1a). The other boundary (−399) of protection against exonuclease III was identified by performing a similar assay using the *Xma*I–*Xba*I fragment labeled at the *Xba*I site at −504 (data not shown). This DNA binding factor was tentatively designated ColF1. Although the exonuclease III assay has the advantage of defining the boundaries of the DNA segment protected by a DNA binding protein, it cannot be easily used for quantitative measurements. We therefore used a gel mobility shift assay with a 72 bp *Hin*dIII–*Eco*RI DNA fragment of plasmid pAH5 labeled at the *Hin*dIII site; this 72 bp fragment contains a cloned oligonucleotide containing the sequence between −419 and −399 of the mouse α2 (I) collagen gene within the polylinker of pUC-9 (Fig. 1b). Specificity of the gel retardation assay is indicated by the fact that the signal is competed by excess amounts of the unlabeled 72 bp *Hin*dIII–*Eco*RI fragment of pAH 5 but not by a control DNA (Fig. 1b). The effect of temperature on the activity present in the crude extract was tested by gel retardation assay. Incubation at 60°C for five minutes resulted in about 50 percent loss of activity which was further reduced after incubation at 95°C for five minutes (data not shown).

NIH-3T3 nuclear extracts were first applied to a col-



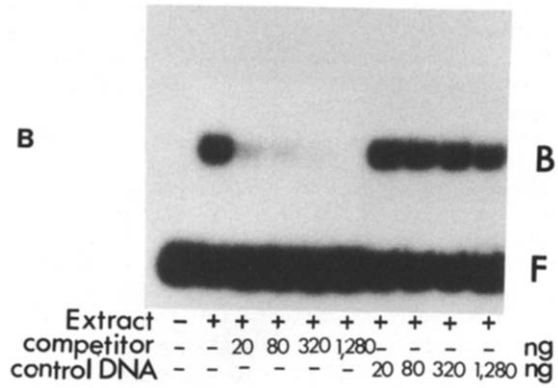


Fig. 1. Exonuclease III and gel retardation assay of the ColF1 binding activity. (A) The upstream border of the binding site of the factor was assayed by exonuclease III. The *Xba*I–*Xma*I fragment of the mouse α2 (I) collagen promoter (−504 to −104), 5′ end-labeled at the *Xma*I site (−104) was incubated with exonuclease III in the absence or presence of nuclear extracts from NIH-3T3 cells (2 mg/ml). Competitor DNA was a 72 bp fragment containing the sequence of the mouse α2 (I) collagen gene between −424 and −394. A 41 bp unrelated *Hin*dIII–*Eco*RI DNA fragment of pUC 12 was used as control competitor. Size markers were end-labeled φX174 DNA fragments digested by *Hae*III. Lanes 1 and 2 have no extraact and each contain two different concentrations of exonuclease III. The arrow indicates a 5′ boundary of protection mapping at −420. (B) A gel retardation assay was performed using 2 μg of protein of a nuclear extract prepared from NIH-3T3 cells. The labeled fragment is a 72 bp *Hin*dIII–*Eco*RI fragment of pAH5 containing the sequence of the mouse α2 (I) collagen promoter between −424 and −394 within a polylinker segment, 5′ end-labeled at the *Hin*dIII end. B represents the protein DNA complex; F free DNA. The indicated amounts of the unlabeled 72 bp *Hin*dIII–*Eco*RI fragment of competitor pAH5 or the 41 bp *Hin*dIII–*Eco*RI of pUC 12 control DNA were included in the binding reaction as marked.

umn of heparin-agarose. The active factor was eluted with 0.75 M NaCl yielding a 5-fold purification (Table I). The major purification step was achieved by using a DNA affinity column prepared from a double-stranded oligonucleotide containing the sequence between −419 and −399 of the mouse α2 (I) collagen promoter (Fig. 2a). When the active heparin-agarose fraction was passed through this DNA affinity column, activity was eluted with a 1 M NaCl step. The active fractions were then passed a second time over the column which was washed with increasing concentrations of NaCl. The majority of the binding activity was eluted with 0.75 M NaCl (Fig. 2b) although a substantial loss of DNA binding activity was observed after the second affinity column. A 500-fold purification was obtained with the first and an additional 6- to 7-fold purification with the second affinity column (Table I). By silver-stain of an SDS-polyacrylamide gel two protein bands of 40 and 42.5 kDa were detected in the fraction eluting at 0.75 M NaCl (Fig. 2c). Additional bands (116 and 55 kDa) were also seen but these did not demonstrate activity in subsequent renaturation experiments (see below). The purified fraction was also used in the exonuclease III assay and showed a signal at the −419 boundary of binding (data not shown). The active fraction obtained after the first DNA affinity chromatogra-

Table I

Purification of ColF1 from NIH-3T3 cells

Fraction	Volume (ml)	Protein (mg)	Specific activity (unit/μg)[a]	Purification (fold)	Yield (%)
Nuclear extract	65	118	0.6	1	100
Heparin-agarose	10	20	3	5	85
First DNA-affinity	1.5	0.012	1,660	2,767	28
Second DNA-affinity	1.5	0.0006	11,100	18,500	9

[a] One unit is defined as the activity causing retardation of 1% of the labeled fragment under the conditions of our assay.

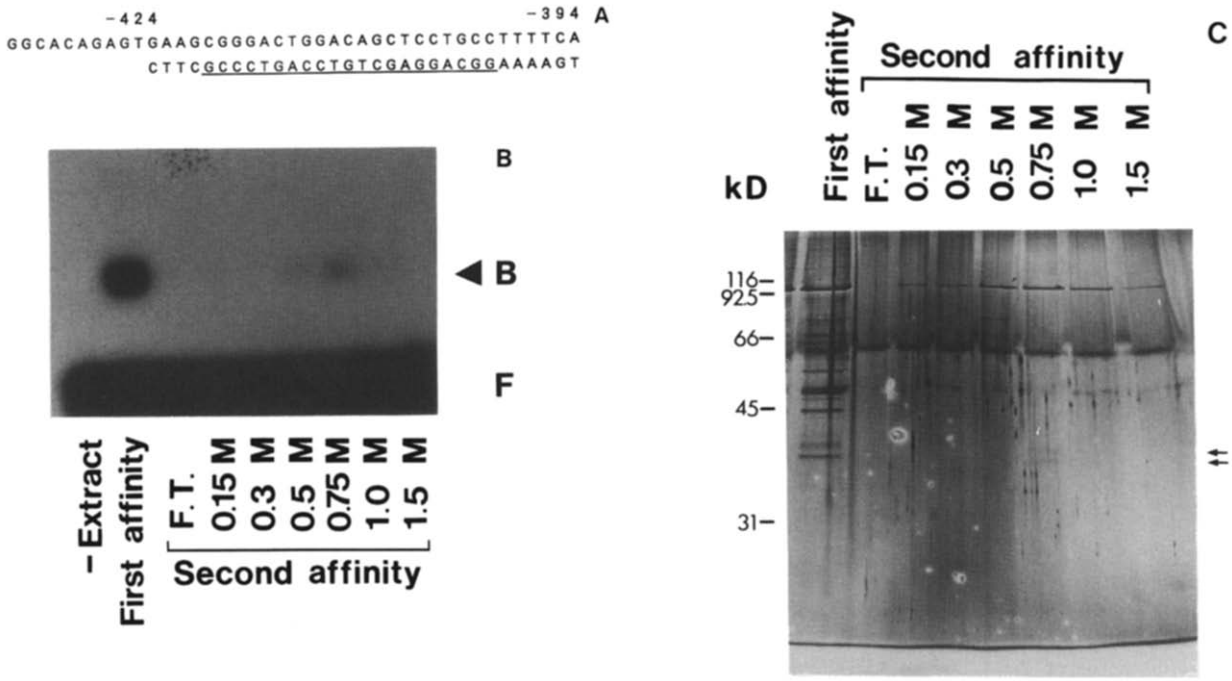


Fig. 2. Purification of the ColF1 binding factor by sequence-specific DNA-affinity chromatography. (A) Sequences of the 41-mer sense strand and the 31-mer anti-sense DNA used for affinity chromatography. The complementary segments of the oligonucleotides correspond to the region from −424 to −394 of the mouse α2 (I) collagen promoter. The sequence protected in the exonuclease III assay is underlined. After hybridization to each other the double stranded oligonucleotide was coupled to CHBr-activated Sepharose 4B. (B) The fraction eluted after heparin agarose chromatography by buffer A containing 0.75 M NaCl, was passed over the affinity column. This fraction was then diluted 20-fold with buffer A without NaCl and applied to a second similar column. This column was then washed with buffer A and consequently eluted with buffer A containing the indicated concentrations of NaCl. One microliter of the fractions was tested for binding activity of ColF1 binding factor using the gel retardation assay. B, DNA–protein complex; F, free DNA; FT, flow through. (C) Another portion (30 μl) of the fractions was analyzed by SDS-polyacrylamide gel and silver staining. The two arrows indicate the position of the 40 and 42.5 kDa bands.

phy was also applied to an FPLC MonoQ column and eluted with a 25 ml linear gradient of NaCl (50 to 500 mM). The peak of ColF1 binding activity was detected at approximately 0.4 M, as determined by gel retardation assay (Fig. 3A). When these fractions were analyzed by silver staining both the 42 kDa and 40.5 kDa polypeptides were detected (Fig. 3B). The amounts of these polypeptides correlated well with the binding activities seen in the gel retardation assay. The binding activity of these purified fractions was also competed by the 72 bp *Hin*dIII–*Eco*RI fragments of pAH5 but not by an unrelated DNA fragment.

In order to provide conclusive evidence that the two major polypeptide species purified by the DNA affinity column bind to the segment between −419 and −399, renaturation experiments were performed. The active fraction obtained after one passage of DNA affinity chromatography was fractionated by electrophoresis on an SDS-polyacrylamide gel. The entire gel was cut into 0.5 cm slices, the proteins were eluted and subjected to renaturation. As shown in Fig. 4A only the region containing both 40 and 42.5 kDa bands exhibited DNA binding activity after renaturation. Furthermore, that region was then examined more precisely as demonstrated in Fig. 3B. Two peaks containing binding activity were found, the first peak contained the 42.5 kDa polypeptide and the second peak contained the 40 kDa polypeptide (Fig. 4b). The retarded band which corresponds to the complex formed with the 42.5 kDa polypeptide migrates slightly slower than the band corresponding to the complex formed with the 40 kDa polypeptide.

We wished to compare the levels of ColF1 binding activity in nuclear extracts from a variety of cell types, which had previously been shown to synthesize different amounts of type I collagen. As shown in Fig. 5A a three to four times reduced level of ColF1 binding activity was found in v-*mos* and v-*ras* transformed NIH-3T3 fibroblast compared to normal NIH-3T3 cells. It was shown previously that the level of type I collagen RNA is reduced in v-*mos* and v-*ras* transformed cells [4]. Similarly a substantial decrease was detected in nuclear extracts from epidermal cells and in extracts from lymphocytes. To provide evidence that these differences in DNA binding activities were not due to general degradation of nuclear proteins, we compared the DNA binding activities of CBF in nuclear extracts of the same cell lines. The results of Fig. 5B show that the extracts of the different cell lines did not contain significant differences in the levels of CBF binding activity.

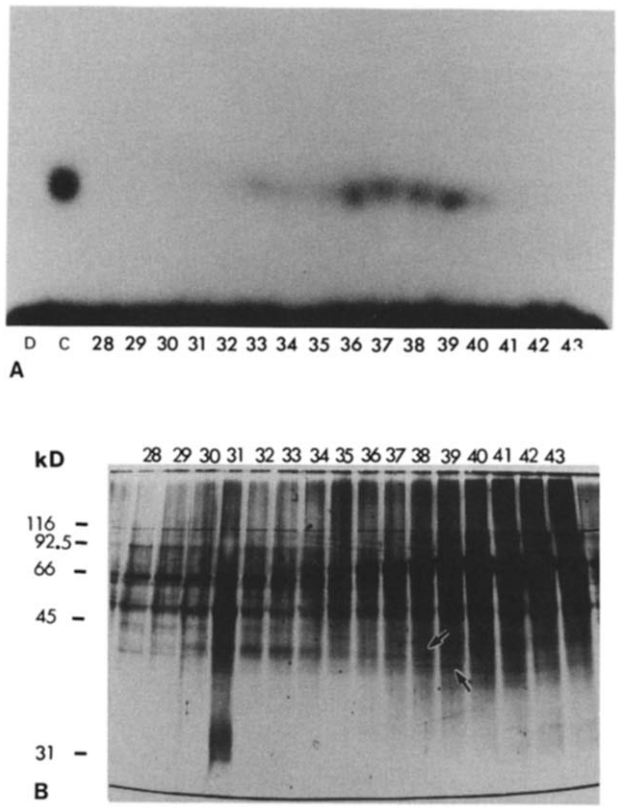


Fig. 3. Characterization of the binding factor by FPLC. Fractions purified once by DNA affinity chromatography were applied to an FPLC Mono Q column and eluted with a 25 ml linear gradient of NaCl (50 to 500 mM). Fifty fractions were obtained, and all the fractions were dialyzed against buffer A. (A) Three μl of samples from each fraction were tested for binding activity by the gel retardation assay. Lane D, DNA probe only; lane C, the protein sample from the first affinity column. (B) Thirty μl of samples from each fraction were analyzed by SDS-polyacrylamide gel and silver staining. The two arrows indicate the 40 and 42.5 kDa bands. The numbers are the fraction numbers.

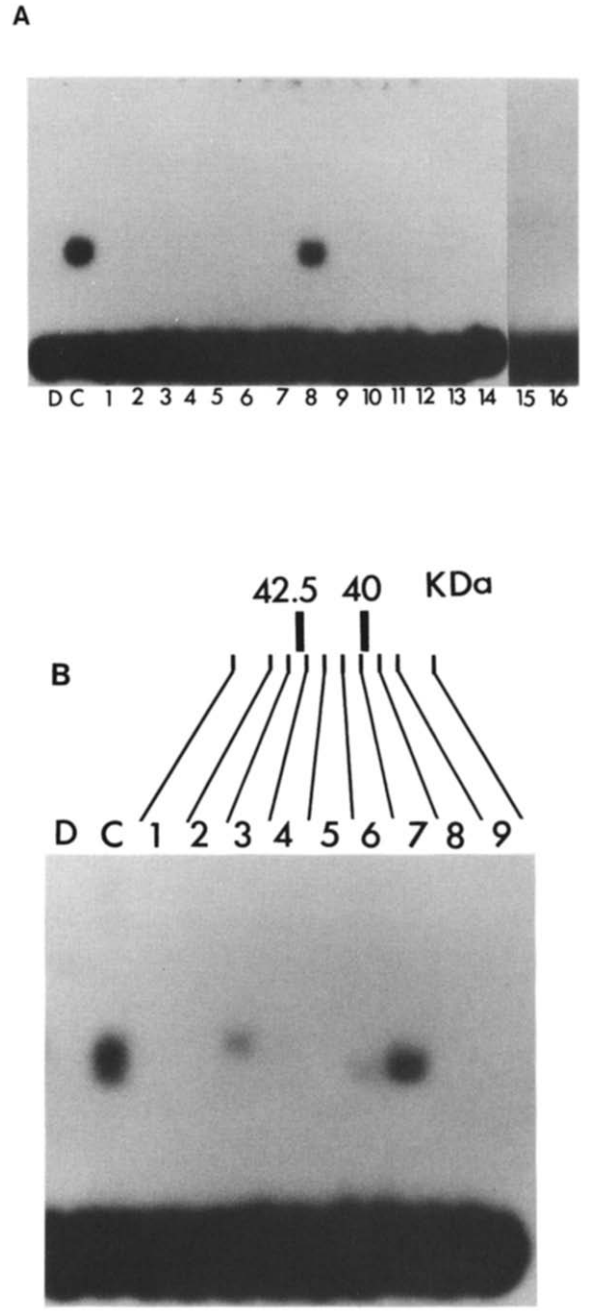


Fig. 4. Renaturation of ColF1 binding activity. Four-hundred microliter aliquots of the fraction purified by one cycle of DNA affinity chromatography were concentrated by 80% acetone precipitation, resuspended in loading buffer and loaded on two 10% SDS-polyacrylamide gels. In each gel 9/10 of the sample was loaded on one lane for renaturation experiments, 1/10 of the sample was loaded on another lane for silver staining. (A) After electrophoresis, the entire gel lane was cut into 0.5 cm slices. (B) The region around the position of slice 8 of gel A was cut into 1 mm slices for slices 1 and 9 and 0.5 mm slices for slices 2 to 8. Renaturation was performed as described by Briggs et al. [23]. Three μl of renatured protein from each slice were tested for binding activity in the gel retardation assay. Lane D, DNA probe only; lane C represents a protein sample from the first affinity column.

Several proteins have previously been identified as factors which are likely to regulate transcription of the mouse of α2 (I) collagen gene [7–11]. We report here on the properties of another protein which binds to a specific site in this promoter. The binding activity of this factor is significantly higher in extracts of cells which synthesize higher levels of type I collagen. The factor present in a nuclear extract from mouse NIH-3T3 fibroblasts protects a segment between −419 and −399 upstream of the transcription start site of the α2 (I) collagen gene. This segment displays an incomplete inverted repeat (5′-GGACTGG/ACAGCTCC-3′) which could eventually represent a binding site for two possible subunits of a homodimer. Alternatively, the segment could simply represent a duplicated site for the same binding protein. The specificity of binding was demonstrated by the ability of an unlabeled fragment containing the same sequence to act as competitor in inhibiting binding to the labeled fragment whereas an unrelated fragment did not compete. Partial homology was seen between this binding site and a segment in the first intron of the human steroid 21-hydroxylase B gene [24].

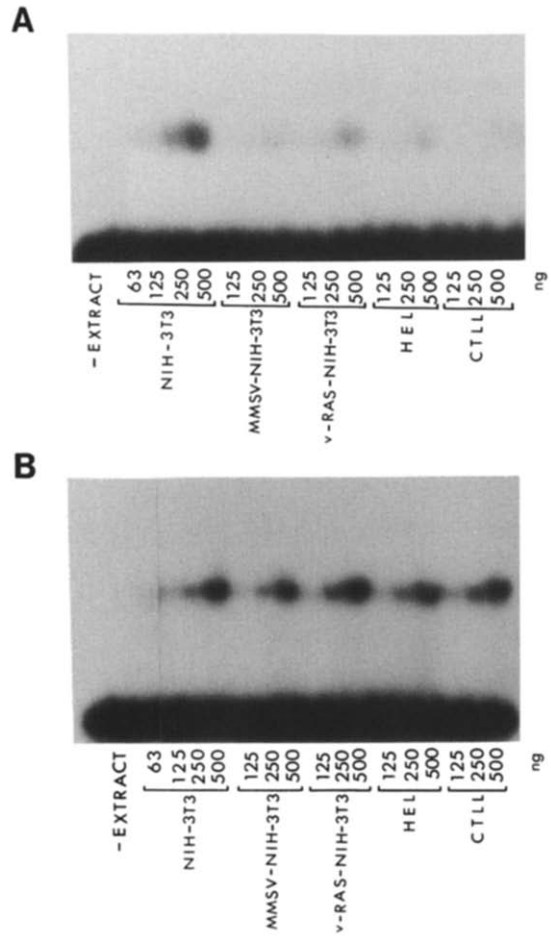


Fig. 5. Gel retardation assay of ColF1 binding factor and CCAAT binding factor (CBF) using nuclear extracts from different cell lines. (A) Indicated amounts of crude nuclear extracts from NIH-3T3 cells, v-*mos* transformed NIH-3T3 cells, v-*ras* transformed NIH-3T3 cells, mouse epidermal cells (HEL cells) and mouse T-lymphocytes (CTLL cell) were assayed for binding activity of ColF1 binding factor by gel retardation assay using the 72 bp ^{32}P-labeled *Hin*dIII–*Eco*RI fragment of pAH5, 5′-end labeled at the *Hin*dIII site. (B) Binding activity of CBF in the same nuclear extracts was assayed by gel retardation using a ^{32}P-labeled *Hin*dIII–*Apa*I fragment (−105 to +54 of the mouse α2 (I) collagen promoter) of pAZ 1003.

No other sequence homologies were found with the −419 and −399 sequence in a computer search of available gene banks.

Purification of the factor from NIH-3T3 fibroblast nuclear extracts revealed two species with molecular weight of 40 and 42.5 kDa, which both exhibited DNA binding activity after elution and renaturation from an SDS-polyacrylamide gel. Using DNase I or exonuclease III footprinting methods, we have previously detected discrete binding sites for DNA binding proteins between +1 and −350 in the α2 (I) collagen promoter, one for CBF between −75 and −95, one for an unknown factor around −250 and one for NF1 around −300. The sequences in these binding sites bear no similarities with the binding site for ColF1. The chromatographic elution pattern of these proteins on heparin agarose are also different. Indeed ColF1 elutes at somewhat higher salt concentrations than CBF and NF1 [8,25]. Moreover, CBF is a heteromer which can be separated in two complementary activities by chromatography on Mono Q [8]. After Mono Q chromatography CBF DNA binding activity can only be restored if two different fractions are combined. Separately, the fractions display no CBF DNA binding activity, whereas ColF1 retains its activity after elution from Mono Q. Furthermore, a DNA fragment containing the CBF binding site did not compete for binding of the ColF1 factor to its binding site. Finally, in crude extracts CBF is less heat stable than the ColF1 binding factor.

In summary, ColF1 is a factor which binds to a specific site in the promoter of the mouse α2 (I) collagen gene. Its DNA binding activity is considerably higher in nuclear extracts from NIH-3T3 fibroblasts than in similar extracts from lymphoid cells and epithelial cells. NIH-3T3 fibroblasts are known to synthesize substantial amounts of type I collagen whereas the two other cell lines do not synthesize type I collagen. cDNA clones for ColF1 and transcription experiments in vivo and in vitro should help in further characterizing the function of this factor.

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