chk-YB-1b, a Y-box binding protein activates transcription from rat $\alpha 1(I)$ procollagen gene promoter

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Type-I collagen, the predominant component of extracellular matrix, is a triple-helical protein consisting of two $\alpha 1$ polypeptides and one $\alpha 2$ polypeptide. Expression of $\alpha 1$ and $\alpha 2$ procollagen genes is co-ordinately regulated under both normal and various pathological conditions. However, the basis of this co-ordinate regulation is not well known. YB-1b, a Y-box protein, has been shown to bind to the polypyrimidine tract present in the $\alpha 2$ procollagen gene. Here, we show that chk-YB-1b, a YB-1 homologue, binds in a single-strand-sequence-specific manner to the highly conserved pyrimidine-rich sequences in both $\alpha 1(I)$ and $\alpha 2(I)$ procollagen promoters from different species, as demonstrated by electrophoretic-mobility-shift assays and by DNaseI footprinting experiments. Transiently transfected and retrovirally expressed antisense oligonucleotides directed against chk-YB-1b specifically inhibited the $\alpha 1(I)$ procollagen promoter-driven transcription in cultured fibroblasts. Considering these data and the fact that the chk-YB-1b binding site is one of the few sites between $\alpha 1(I)$ and $\alpha 2(I)$ procollagen promoters that is conserved from chicken to human, it is proposed that chk-YB-1b may be involved in co-ordinate expression of these two collagen genes.

INTRODUCTION

Type-I collagen, the predominant component of the extracellular matrix, is a heterotrimeric molecule consisting of two polypeptide chains of COL1A1 and one polypeptide chain of COL2A1, which are encoded by two different genes, $\alpha 1(I)$ and $\alpha 2(I)$, respectively. Transcripts for $\alpha 1(I)$ and $\alpha 2(I)$ procollagen are synthesized at a 2:1 ratio, indicating that these two genes are co-ordinately regulated at the transcriptional level (reviewed in [1]). Regulation of transcription of genes encoding α1(I) and α2(I) collagen has been the subject of several investigations [1–6]. Transgenic mice experiments using mouse $\alpha 1(I)$ or $\alpha 2(I)$ procollagen gene promoters have shown that up to 476 bp for $\alpha 1(I)$ [7] and 350 bp for $\alpha 2(I)$ [8] upstream sequences are sufficient to confer tissue-specific expression. However, neither the *cis*-acting elements nor their cognate transcription factors involved in this co-ordinate regulation have been fully characterized.

Examination of the sequence of the promoter regions up to -200 bp indicated that only one polypyrimidine/polypurine tract, of about 30 bp, is highly conserved between $\alpha 1(I)$ and $\alpha 2(I)$ procollagen promoters from chickens to humans. Whether this motif is involved in co-ordinate expression of transcription of both collagen genes is not known. The polypyrimidine tract in the mouse $\alpha 2(I)$ collagen gene has been shown to contain S1 nuclease-sensitive sites, suggesting that this region exists in a single-stranded structure *in vivo*. A looping-out model generated by slippage of the repeat sequence has been proposed to explain the existence of S1 nuclease-sensitive sites [9]. Alternatively, these polypyrimidine tracts could form intramolecular triple-helix or H-structures, as demonstrated for the decorin gene promoter, thus exposing unpaired regions that are sensitive to S1 nuclease [10]. Deletion and mutation analyses indicated that this polypyrimidine tract is important for collagen gene regulation [11–13].

In our laboratory we have cloned a transcription factor, chk-YB-1b [14], which belongs to a large family of regulatory proteins collectively called Y-box proteins [15]. These proteins share a highly conserved cold-shock domain that is absolutely necessary for DNA binding. A variety of functions, including transcriptional activation or repression [16,17], chromatin modification [18], translational masking of RNA [19] and RNA packaging [15] have been attributed to Y-box proteins. Several Y-box proteins are known to preferentially bind pyrimidine-rich sequences [20–22]. Members of this family of proteins are known to interact with both double-stranded and single-stranded DNA as well as with RNA [17,23,24].

Recently it has been shown that chk-YB-1b, a chicken homologue of human YB-1, binds to the pyrimidine-rich sequence in the chicken $\alpha 2(I)$ procollagen promoter [25]. In the present study, we show that chk-YB-1b also binds to the single-stranded polypyrimidine tract present in both $\alpha 1(I)$ and $\alpha 2(I)$ promoters of chicken and mammalian collagen genes. Using antisense oligodeoxyribonucleotides (ODNs) transfected transiently, or stably expressed via infection with retroviral constructs, we show that blocking the expression of YB-1b results in down-regulation of procollagen $\alpha 1(I)$ transcription. Based on these results, we propose that YB-1b may have a role in the co-ordinate expression of $\alpha 1(I)$ and $\alpha 2(I)$ procollagen genes.

MATERIALS AND METHODS

Cell cultures

Chicken fibroblasts (CKY) were grown in medium 199 (M199) supplemented with 0.22% $NaHCO_3$/10% fetal calf serum (Hyclone, Logan, UT, U.S.A.)/10% tryptose phosphate broth/1% chicken serum/100 units/ml of penicillin/10 μg/ml of streptomycin in a humidified 5% CO_2 incubator at 37 °C. Rat 2tk$^-$

Abbreviations used: ODN, oligodeoxyribonucleotide; EMSA, electrophoretic-mobility-shift assay; CAT, chloramphenicol acetyl transferase; UTR, untranslated region; H-DNA, cruciform, triple-helical DNA.

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and PA317 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 0.37% $NaHCO_3$, 10% calf serum (Hyclone), 100 units/ml of penicillin and 10 μg/ml of streptomycin.

ODN synthesis and preparation

Several phosphorothioate-modified 21-mer sense and antisense ODNs corresponding to *chk-YB-1b* coding, as well as 3′ untranslated regions (UTRs), were obtained from Oligonucleotides Therapeutics Inc. (Wilsonville, OR, U.S.A.). All other ODNs used in this study were synthesized in an Applied Biosystems 381A DNA synthesizer at the DNA Core Laboratory of the University of Missouri-Columbia. Double-stranded ODNs were prepared by mixing equal amounts of complementary single strands in the presence of 0.3 M NaCl. The mixture was heated to 80 °C for 5 min, incubated at 55 °C for 1 h and then at 42 °C for 1 h. The resulting double-stranded oligonucleotides were used as such or after gel purification.

Electrophoretic mobility shift assays (EMSAs)

Partially purified Mal-chk-YB-1b fusion protein, cleaved by factor Xa, was prepared as described previously [14]. Increasing amounts of this protein were incubated with about 25000 c.p.m. (0.1–0.2 ng) of end-labelled ODNs in a final volume of 15 μl for 25 min at room temperature. All binding reactions contained 25 mM Tris/HCl (pH 7.5), 0.05 mM EDTA, 60 mM KCl, 1 mM dithiothreitol, 5% glycerol and 2 μg of poly(dI-dC). In competition experiments, excess unlabelled ODNs (competitor) were incubated with protein for 5 min before adding the labelled probe. Following electrophoresis in a 6% polyacrylamide gel (acrylamide/bis at 39:1) in Tris-glycine-EDTA buffer [37 mM Tris/HCl (pH 8.0)/50 mM glycine/2 mM EDTA] at 10 V/cm, gels were dried and autoradiographed at −70 °C.

DNase I footprinting

For DNaseI footprinting of the rat $\alpha 1(I)$ procollagen promoter, the single-stranded DNA probe was generated as follows. The −214 to −69 bp region of the rat $\alpha 1(I)$ collagen promoter was amplified by PCR using a radiolabelled forward primer (214F, 5′-CAAGGGTGGCAGAATTGCAA-3′) and a non-labelled reverse primer (−69R, 5′-GGAGAGGGGGAGCCAGCAGC-3′). The PCR product was purified by extraction with phenol/chloroform and precipitation with ethanol. The pellet was finally dissolved in 0.1 M NaOH to keep the −214 to −69 bp PCR product denatured. The denatured probe in which the positive strand was labelled at the −214 position was incubated with increasing amounts of chk-YB-1b for 20 min and then digested with 25–50 ng of DNaseI for 1 min at room temperature. The reaction was stopped by adding 50 μl of stop solution containing 200 mM NaCl, 20 mM EDTA, 1% SDS and 250 μg/ml yeast tRNA. Samples were extracted once with phenol, once with phenol/chloroform (1:1), and then ethanol precipitated. Precipitates were collected by centrifugation, dried, and resuspended in 70% formamide buffer. Samples were then heat-denatured and loaded on to an 8% acrylamide/7 M urea gel. Gels were dried and autoradiographed at −70 °C with an intensifying screen.

Transient transfections

Chick embryo fibroblasts in the mid-log phase of growth were transfected with plasmid DNA using LipofectAMINE (Gibco-BRL, Grand Island, NY, U.S.A.), according to the manufacturer's protocol. Briefly, plasmid DNA or ODNs were pre-incubated with LipofectAMINE in 300 μl of serum-free M199 at room temperature for 45 min. Cells were washed with pre-warmed serum-free M199. The DNA–LipofectAMINE mixture was diluted in M199 to a final volume of 3 ml and added to the plates. Cells were then incubated at 37 °C for 6 h, after which the medium was exchanged with complete M199. Cells were first transfected with 10 μg of either control, sense or antisense ODNs followed by another transfection with 10 μg of reporter plasmid pColCat330. Plasmid pColCat330 contains the −330 to +100 bp region of the rat $\alpha 1(I)$ procollagen promoter [26]. pSVgal plasmid (3 μg; Promega, Madison, WI, U.S.A.) encoding β-galactosidase was co-transfected to normalize transfection efficiency. After 48 h, cells were harvested by scraping in 1 ml of 40 mM Tris/HCl (pH 7.5)/1 mM EDTA/150 mM NaCl. Cell extracts (50 μg of protein) were assayed for β-galactosidase and chloramphenicol acetyl transferase (CAT) as described previously [26].

Stable retroviral transfections and Northern-blot analysis

A 462 bp PCR product corresponding to +430 to +892 bp of *chk-YB-1b* cDNA [14] was generated and cloned in both orientations into the pLNCX retroviral vector [27], which harbours a neomycin-resistance cassette. Clones expressing *YB-1b* in sense or antisense orientations were determined by restriction digests and transfected into the amphotropic mouse fibroblast packaging cell line PA317. Stable transfectants were selected by treating cells with G418 (400 μg/ml) and virus collected from the cell culture medium, and used to infect rat $2tk^-$ fibroblasts plated at 1×10^5 cells per plate. Stable transfectants were then amplified by G418 selection and total RNA was isolated, quantified, and 5 μg per lane was separated on 1.2% agarose/formaldehyde gels. RNA was then vacuum-transferred to nylon membranes, pre-hybridized for 1 h at 55 °C in 10 ml of hybridization buffer [50 mM Pipes/100 mM NaCl/50 mM sodium phosphate (pH 7.0)/1 mM EDTA], and probed for 12 h at 55 °C in 5 ml of hybridization buffer with 1×10^6 c.p.m./ml of $\alpha 1(I)$ or $\alpha 2(I)$ rat collagen or 18 S rRNA cDNA probes. Blots were first washed with 50 ml of 5% SDS and 1 × SSC (0.15 M NaCl/0.015 M sodium citrate) for 10 min at room temperature, then with another 50 ml for 20 min at 55 °C, and were then dried and autoradiographed at −70 °C.

RESULTS

chk-YB-1b binds to multiple sites within the collagen $\alpha 1(I)$ promoter

chk-YB-1b (cloned and purified in our laboratory) is known to bind to a pyrimidine-rich sequence located around −200 bp in the chicken $\alpha 2(I)$ procollagen promoter [25]. Since a highly conserved polypyrimidine sequence is also present in the rat $\alpha 1(I)$ promoter, we tested binding of chk-YB-1b to the rat sequence by EMSAs, using both single- and double-stranded ^{32}P-labelled −214 to −69 bp segments of the rat $\alpha 1(I)$ procollagen promoter as the probe. Our results indicate that chk-YB-1b binds strongly to single-stranded DNA, but not to the double-stranded DNA probe (Figure 1). Since almost all the single-stranded DNA probe existed as a bound complex when higher concentrations of chk-YB-1b were used, we suggest that this factor binds to both strands of the DNA. It should also be noted that with increasing amounts of protein in the reaction, more than one DNA–protein complex was observed, suggesting that either the protein binds to multiple sites on the collagen promoter or that the complexes may represent multimers of YB-1b, as reported earlier for

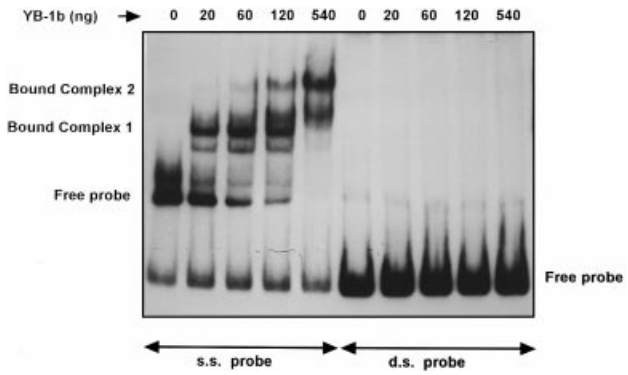


Figure 1 chk-YB-1b binds to multiple sites on the rat type-I $\alpha 1(I)$ collagen promoter

PCR amplification in the presence of [α-^{32}P]dATP and other non-labelled nucleotides was used to label the rat $\alpha 1(I)$ collagen promoter fragment from −216 to −69 bp. This fragment was then used in EMSAs in either a denatured (s.s.; lanes 1–5, from left) or a double-stranded (d.s.; lanes 6–10, from left) form, with increasing amounts of chk-YB-1b. Various DNA–protein complexes, which appeared with progressively higher amounts of protein when the single-stranded probe was used, are marked.

FRGY1 and FRGY2 at higher concentrations [28]. Preferential binding to single-stranded DNA has also been shown for other Y-box proteins, like dbp-B, NSEP-1, BP-8, cspB and chk-YB-2 [16,20,23,29].

In order to identify the sequence motif recognized by chk-YB-1b in the rat $\alpha 1(I)$ procollagen promoter, DNase I footprinting was performed using a single-stranded DNA end-labelled at −214 bp (−214 to −69) of the positive strand as described in Materials and methods. Three distinct areas of protection (marked as a, b and c in Figure 2) were observed when this collagen promoter DNA fragment was incubated with chk-YB-1b (Figure 2, lanes 2–4). The protected area a corresponds to a motif in the polypyrimidine sequence (−140 to −170 bp, or C1 region), whereas areas b and c are part of the polypurine sequence (−170 to −200 bp, or C2 region) distal to the polypyrimidine region (also see [13,30]). Here we would like to point out that binding site a encompasses the polypyrimidine sequence of the $\alpha 1(I)$ and $\alpha 2(I)$ procollagen promoters, which is highly conserved among different species (see Figure 4, lower panel). These results further confirm binding studies (see below) and demonstrate the presence of multiple binding sites within the −214 to −69 bp region of the $\alpha 1(I)$ collagen promoter.

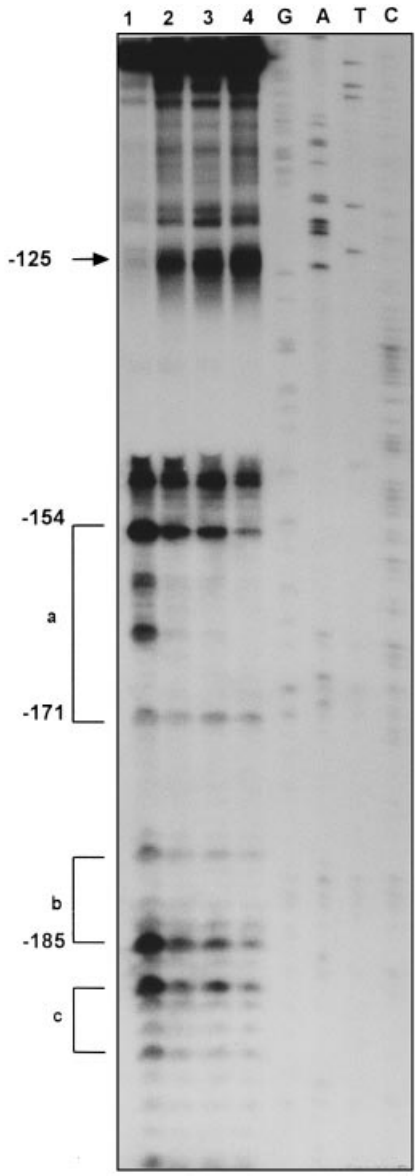


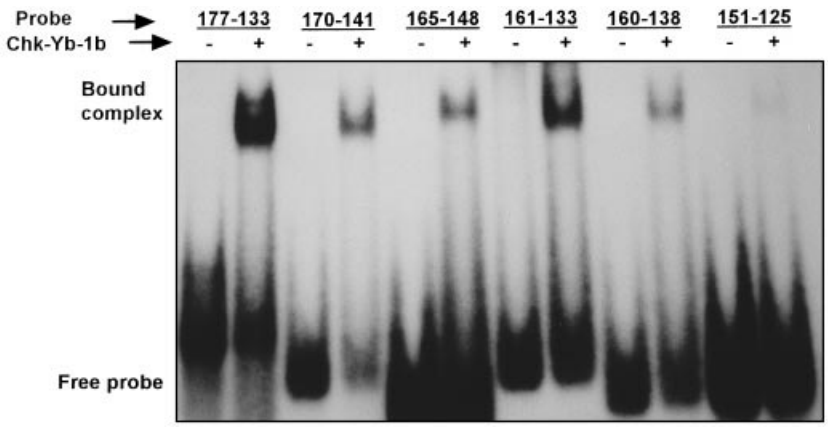
Figure 2 DNaseI footprint of the rat $\alpha 1(I)$ procollagen promoter

The single-stranded −216 to −69 bp fragment labelled at the −214 site, as described in Materials and methods, was incubated without chk-YB-1b (lane 1), or with 50 ng (lane 2), 100 ng (lane 3) or 200 ng (lane 4) of chk-YB-1b, and then subjected to DNaseI footprinting. Numbers on the left correspond to bp upstream of the transcription initiation site. Regions protected from DNaseI digestion are marked as a, b and c.

chk-YB-1b binds to the polypyrimidine sequence in the $\alpha 1(I)$ collagen promoter

The 35-bp long polypyrimidine sequence from −172 to −138 bp is highly conserved among type-I procollagen promoters. One of the areas protected in DNA footprinting experiments (marked as a in Figure 2) corresponds to this polypyrimidine sequence. To further localize the sequence motif recognized by chk-YB-1b, we used shorter oligonucleotides representing this region in EMSAs. A single-stranded ODN from −177 to −133 bp (corresponding to protected area a) was end-labelled and incubated with chk-YB-1b. The results shown in Figure 3 indicate binding of chk-YB-1b to this ODN with high affinity. The complementary polypurine single-stranded ODN showed very weak binding to chk-YB-1b and no binding was seen with double-stranded ODNs (results not shown). To further narrow down the region responsible for chk-YB-1b binding, we made several deletions from both ends of the −177 to −133 ODN and carried out EMSAs. The results of representative EMSAs and a summary of results obtained from several such studies are shown in Figure 3. Truncating the −177 to −133 ODN from either end resulted in decreased binding with YB-1b. The shortest ODN that showed significant binding with chk-YB-1b was from −161 to −133 bp. In order to determine the sequence specificity of the interaction between chk-YB-1b and the −177 to −133 ODN, we made mutant oligonucleotides containing 4–6 bp substitutions. All the mutant ODNs (M1–M3) competed effectively with the wild-type ODN for chk-YB-1b binding. These results suggest that chk-YB-1b appears to have a broader sequence specificity, especially within the polypyrimidine tract (Figure 3).

(A)



(B)

Oligonucleotide	Sequence	Binding
-177-133	5′ GTGGACTCCTTTCCCTTCCTTTCCCTCCTCCCCCCTCTTCGTTCC 3′	++++
-177-133 C	3′ CACCTGAGGAAAGGGAAGGAAAGGGAGGAGGGGGGAGAAGCAAGG 5′	+
-177-148	5′ GTGGACTCCTTTCCCTTCCTTTCCCTCCTC 3′	++
-177-160	5′ GTGGACTCCTTTCCCTTC 3′	-
-172-148	5′ CTCCTTTCCCTTCCTTTCCCTCCTC 3′	++
-170-141	5′ CCTTTCCCTTCCTTTCCCTCCTCCCCCCTC 3′	++
-170-141 C	3′ GAGGGGGGAGGAGGGAAAGGAAGGGAAAGG 5′	+
-165-148	5′ CCCTTCCTTTCCCTCCTC 3′	+
-162-145	5′ TTCCTTTCCCTCCTCCCC 3′	-
-161-133	5′ TCCTTTCCCTCCTCCCCCCTCTTCGTTCC 3′	+++
-160-138	5′ CCTTTCCCTCCTCCCCCCTCTTC 3′	+
-151-125	5′CCTCCCCCCTCTTCGTTCCAAATTGGG 3′	-
-177-133 M1	5′ GTGGACGCCTTTCCATTCATTTCACTCCTCCCCCCTCTTCGTTCC 3′	++++
-177-133 M2	5′ GTGGACTCCTTTCCCTTCCTTTCCCTGGTCCGCCCTCTTCGTTCC 3′	++++
-177-133 M3	5′ GTGGACTCCTTTCGCTTCCTTTCGCTGGTCCGGCCTCTTCGTTCC 3′	++++
-200-171	5′ TTGCAAAGGGGGGAGGGGGCTGGGTGGACT 3′	+
-200-171 C	5′ AACGTTTCCCCCCTCCCCCGACCCACCTGA 3′	+++

Figure 3 Interaction of chk-YB-1b with various upstream ODNs

(**A**) A representative gel showing EMSAs with various ODNs. EMSAs were performed as descibed in the Materials and methods section, with 50 ng of chk-YB-1b. (From left) lanes 1, 3, 5, 7, 9 and 11, no protein. Lanes 2, 4, 6, 8, 10 and 12, 50 ng of chk-YB-1b added. (**B**) Summary of the interaction of chk-YB-1b with various ODNs. $++++$, binding here refers to the binding with an intensity obtained with the wild-type -177 to -133 ODN. Binding with different mutant ODNs is represented relative to this level. $+$, refers to a weak binding. C, complementary; M, mutant.

The second and third regions protected from DNaseI cleavage (b and c in Figure 2) fell within the polypurine tract between -171 and -200 bp of the rat $\alpha 1(I)$ collagen promoter. In order to test whether chk-YB-1b interacts in a sequence-specific manner with one of the strands in this region, we performed EMSAs with ODNs representing either the positive strand or its complementary strand from this region. As can be seen in Figure 3(B), chk-YB-1b was found to bind the polypyrimidine negative strand with higher affinity. These results would explain binding of chk-YB-1b to both strands as suggested by the data presented above (Figure 1).

The Y-box family proteins were initially described based on their binding specificity to inverted CCAAT sequences (reviewed in [15]). There are two inverted CCAAT boxes within the promoter sequence of rat $\alpha 1(I)$ procollagen gene, one at -129 to -125 bp, just downstream of the polypyrimidine stretch, and the other at -100 to -105 bp. Chk-YB-1b did not bind to the -151 to -125 ODN, which contains an inverted CCAAT motif (Figure 3, lanes marked 151–125). These results are consistent with our earlier results and other reports in which Y-box-binding proteins were shown to have greater affinity for pyrimidine-rich sequences as compared with the CCAAT motif [14,23,31,32].

chk-Yb-1b also binds to the $\alpha 2(I)$ collagen promoter

A comparison of the sequences of $\alpha 1(I)$ and $\alpha 2(I)$ promoters from different species shows a conserved polypyrimidine sequence located in the upstream sequence. Therefore, we synthesized 30-mer oligonucleotides, representing the conserved polypyrimidine tract from rat $\alpha 1(I)$, human $\alpha 1(I)$, chicken $\alpha 2(I)$ and rat $\alpha 2(I)$ collagen promoters (Figure 4, bottom panel), and retardation assays were carried out using 50–100 ng of chk-YB-1b protein. These results show that chk-YB-1b binds to the polypyrimidine tract from both $\alpha 1$ and $\alpha 2$ collagen promoters with comparable affinity (Figure 4, top panel). The mouse promoter shows 100%

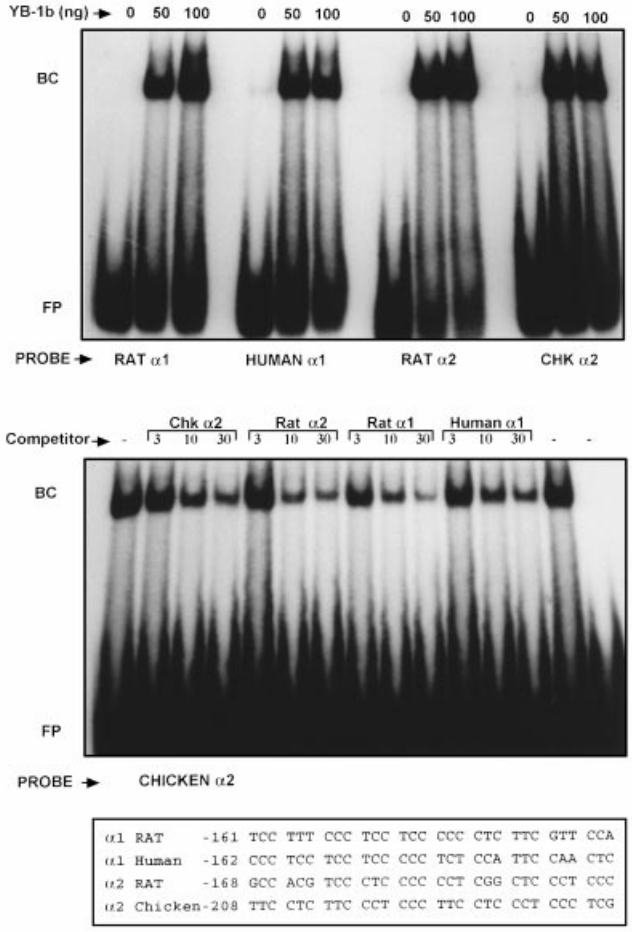


Figure 4 Interaction of chk-YB-1b with *α1(I)* and *α2(I)* procollagen promoter polypyrimidine sequences

Top panel: different single-stranded oligonucleotides representing the rat *α1(I)*, human *α1(I)*, rat *α2(I)* and chicken *α2(I)* procollagen polypyrimidine regions, whose sequences are shown in the bottom panel, were incubated with either 0, 50 or 100 ng of chk-YB-1b, and EMSAs performed as described in Materials and methods. Middle panel: cross competition of binding of chk-YB-1b to chicken (Chk) *α2(I)* polypyrimidine ODN with 3-, 10- or 30-fold molar excess of different non-labelled ODNs as shown above. BC, bound complex; FP, free probe. Bottom panel: sequences of different ODNs used in binding and competition experiments.

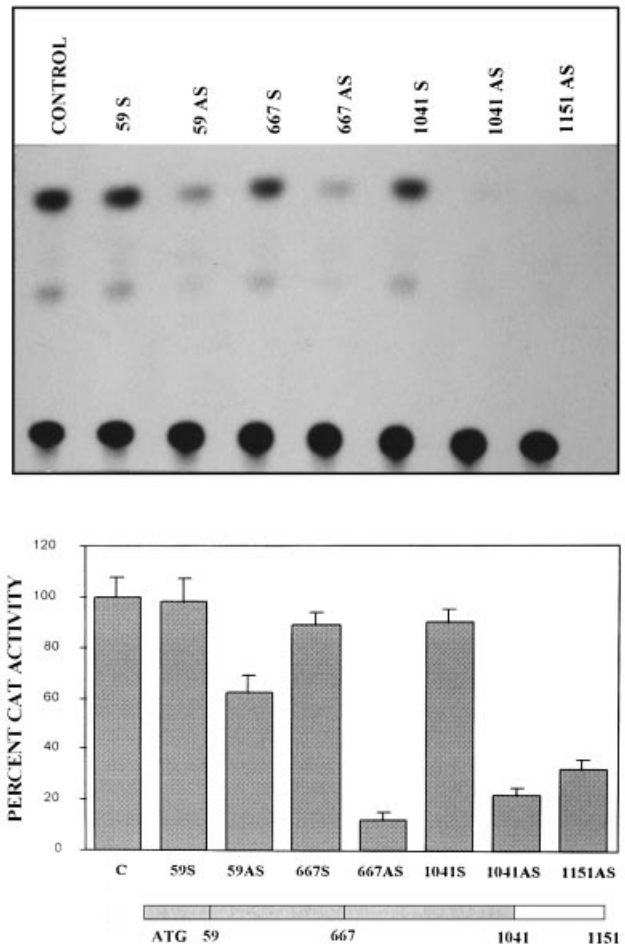


Figure 5 Inhibition of rat *α1(I)* procollagen promoter-driven transcription by antisense ODNs for chk-YB-1b in chick embryo fibroblast cells

pColCAT330 reporter construct was transfected into the chick embryo fibroblast cells after the cells were subjected to an initial transfection with different sense or antisense ODNs for chk-YB-1b. Top panel: a representative chromatograph showing the reporter-gene activity as influenced by either sense (59S, 667S, 1041S) or antisense (59AS, 667AS, 1041AS, 1151AS) ODNs targeted to chk-YB-1b. Lower panel: mean reporter-gene activity (±S.E.M.) obtained from five similar, independently performed, experiments expressed relative to the activity of the pColCAT330 with control ODN. Sequence of ODNs used (5′–3′): control, TGGATCCGACA-TGTCAGA; 59S, CGCCGACTCCAAACCTAACGG; 59AS, CCGTTAGGTTTGGAGTCGGCG; 667S, GGCAGACCAGTCAGGCAGAA; 667AS, TTCTGCCTGACTGGTCTGCC; 1041S, CCAGCAATAAG-AAATGAACA; 1041AS, TCTTCATTTCTTATTGCTGG; 1151AS, TAAAAACCCCATGCTGCATAG.

homology with the rat *α1(I)* promoter sequence used for the retardation assays, and we therefore predict that the mouse promoter would also be bound by chk-YB-1b. In order to determine the specificity of the DNA–protein interactions described above, we performed cross-competition experiments using unlabelled ODNs from rat *α2(I)*, human *α1(I)* and rat *α1(I)* to compete with the chk-YB-1b binding of ^{32}P-labelled chicken *α2(I)* oligonucleotide (Figure 4, middle panel). All the ODNs were equally effective in inhibiting the formation of retarded complex. These observations clearly demonstrate that chk-YB-1b binds to the highly conserved polypyrimidine sequence in both the *α1* and *α2* promoters of type-I collagen and thus may have a role in the co-ordinate expression of these two genes.

The role of chk-YB-1b in collagen gene transcription

As alluded to above, chk-YB-1b is a highly conserved protein. The polypyrimidine sequence present in the promoters of *α1(I)* and *α2(I)* procollagen genes, to which the factor binds, is also well conserved among vertebrates. Therefore, inhibition of expression of *chk-YB-1b* should in principle affect transcriptional activation from any of the type-I procollagen promoters within fibroblast cell lines. In order to understand the functional significance of *in vitro* binding of chk-YB-1b to the *α1(I)* collagen promoter, we studied the effect of chk-YB-1b on collagen transcription in fibroblasts using antisense ODNs to inhibit chk-YB-1b. Specific sense and antisense ODNs corresponding to three different regions of the *chk-YB-1b* mRNA were designed. Chick embryo fibroblasts were first transfected with sense or antisense ODNs followed by another transfection with plasmid pColCat330, which contains the −330 to +115 bp sequence of the rat *α1(I)* promoter region placed upstream of a CAT reporter gene, and the *β*-galactosidase reporter plasmid pSVgal as an internal control [26]. After 48 h, cells were harvested and CAT activity measured. The results (Figure 5) show a 30–80 % decrease

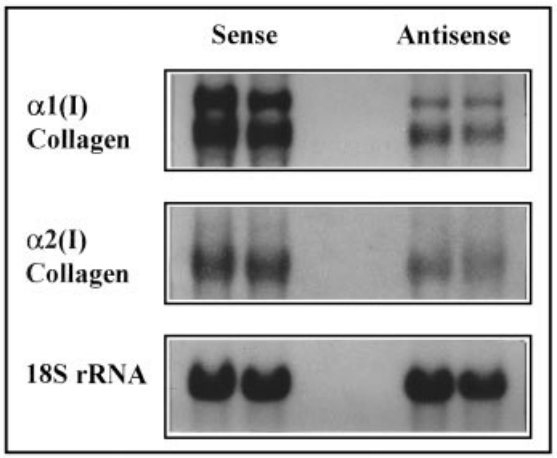


Figure 6 Inhibition of rat *α1(I)* and *α2(I)* procollagen expression by retrovirally expressed antisense RNAs for chk-YB-1b in rat 2tk⁻ fibroblasts

Retrovirus particles containing sense and antisense chk-YB-1b inserts were packaged and used to infect rat 2tk⁻ fibroblasts as described in Materials and methods. RNA was isolated and duplicate samples (5 μg/lane) used for Northern-blot analysis. The blot was hybridized with *α1(I)* and *α2(I)* collagen cDNA probes. An 18 S rRNA probe was used as an internal control.

in CAT activity in cells treated with various antisense ODNs as compared with cells treated with control or sense ODNs. Maximum inhibitory effect was seen with the two antisense ODNs, 667AS and 1041AS, where the CAT activity was reduced by more than 70%. It should be noted that ODNs 1041AS and 1151AS are specific to the 3′ UTR of *YB-1b* mRNA and that both of these ODNs significantly inhibited CAT activity. It is possible that the effect of these ODNs is mediated by binding to the region responsible for stabilizing mRNA. The antisense ODNs did not have any effect on galactosidase activity.

In order to determine the effect of down-regulation of chk-YB-1b on the expression levels of the endogenous *α1(I)* and *α2(I)* procollagen genes, an alternative antisense approach was used. Sense and antisense *YB-1b* inserts were cloned into retroviral constructs, which were packaged into infectious virions and used to infect rat 2tk⁻ fibroblasts, as described in the Materials and methods section. Fibroblasts harbouring stable viral integrants were selected and rat *α1(I)* and *α2(I)* collagen mRNA levels were determined by Northern-blot analysis. Results shown in Figure 6 clearly demonstrate down-regulation of both *α1(I)* and *α2(I)* collagen transcripts from cells infected with retrovirus expressing antisense compared with sense *YB-1b* inserts. These results, in conjunction with the above transient transfection data, strongly implicate chk-YB-1b as a positive regulator of type-I collagen gene expression.

DISCUSSION

In the present study we show that a Y-box protein, chk-YB-1b, binds efficiently to the polypyrimidine tract found in both *α1(I)* and *α2(I)* promoters. chk-YB-1b binds to multiple sites within the *α1(I)* collagen promoter segment from −214 to −69 bp, which includes two polypyrimidine tracts at −200 to −171 bp (C2) and −170 to −147 bp (C1) [30]. Interestingly, the upstream polypyrimidine tract (C2) is in the non-coding strand, whereas the downstream polypyrimidine tract (C1) is in the coding strand [30]. chk-YB-1b binds with comparable affinity to the corresponding well-conserved polypyrimidine tracts in both *α1(I)* and *α2(I)* collagen promoters from rat, mouse, human and chicken.

Y-box proteins typically bind to inverted CCAAT box sequences [15]. However, in addition to binding to the Y-box, these proteins are also known to interact with other promoter elements that exhibit strong purine/pyrimidine asymmetry [19,32]. The early members of the Y-box family of proteins were characterized through their interaction with duplex DNA containing Y-box motifs. However, recent studies have shown Y-box proteins to bind to single-stranded DNA and/or RNA with a higher affinity [17]. In the present study, we have shown that chk-YB-1b binds only to single-stranded DNA but not to double-stranded DNA. This raises an interesting question regarding the existence *in vivo* of single-stranded regions in the collagen promoter. Transcriptionally active genes have been shown to contain S1 nuclease-sensitive sites that often appear as gaps between nucleosomes within chromatin. When under the stress of bending or supercoiling, these sites release histones or other DNA-binding proteins to form 'open' sequences that are free to adopt non-B-DNA structures (i.e. cruciform, triple-helical H-DNA) at polypurine/pyrimidine stretches, which are then accessible to transcriptional regulatory proteins [17]. The Y-box protein NSEP1 binds to pyrimidine-rich single-strands [23], and another cold-shock domain protein has been shown to bind to H-DNA within the γ-globin gene [29]. YB-1b itself has been shown to promote the formation or stabilization of single-stranded regions in the MHC class-II *DR-A* gene promoter [33]. Of particular interest here is the fact that the c-*myc* promoter forms an S1 nuclease-sensitive H-DNA structure that, when formed, directly correlates with the strength of the promoter [34].

The existence of S1 nuclease-sensitive sites within the chicken *α2(I)* gene and the interactions of chk-YB-1b with these single-stranded sequences has been demonstrated [25]. In addition, both the mouse and chicken *α2(I)* genes were shown to contain pyrimidine-rich S1 nuclease-sensitive sites, around −150 and −190 bp, that probably form stem–loop or H-DNA structures [9]. We have identified three major S1 nuclease-sensitive sites within the rat and human *α1(I)* procollagen promoters, mapping to around −190, −170 and −130 bp (results not shown). These S1 nuclease-sensitive sites fall within the regions protected from DNaseI digestion by chk-YB-1b, suggesting that chk-YB-1b-binding sites on the rat *α1(I)* collagen promoter have the potential to form single strands when present on negatively supercoiled plasmids, and may be contributing to the transcriptional regulation of type-I collagen genes.

Antisense ODNs offer the potential to repress or block the expression of specific genes within cells [35,36]. We have used phosphorothioate-modified sense and antisense ODNs in our transfection experiments. The antisense ODNs specific to four different regions of the chk-YB-1b mRNA had a significant inhibitory effect on collagen-promoter-driven transcription, whereas the sense oligonucleotides had no significant effect. ODNs 1041 and 1151 inhibited collagen-promoter activity even though these ODNs were designed to target the 3′ UTR of mRNA. Regulation of mRNA stability has emerged as an important mechanism for regulating cellular mRNA levels [37]. The 3′ UTR interacts with regulatory proteins which may affect the stability of mRNA and thus its half-life. The inhibitory effect of the 1041 and 1151 antisense ODNs targeting the UTR of *YB-1b* mRNA may be due to the role of the 3′ UTR in stabilizing mRNA. It should be noted here that both sense and antisense ODNs had no effect on β-galactosidase activity, which was co-transfected with pColCat330, suggesting that the effect of YB-1b is specific to the collagen promoter.

Earlier work by Karsenty and de Crombrugghe [12] showed that similar oligonucleotide sequences within the mouse *α1(I)* and *α2(I)* procollagen promoters corresponding to the −177 to

−133 bp region bind nuclear extract from NIH 3T3 cells. The factor binding to these sequences was identified as IF-1. Although chk-YB-1b binds to similar sequences, we have evidence to show that IF-1 and chk-YB-1b are not the same factors: (i) IF-1 binds to double-stranded ODNs, whereas chk-YB-1b binds only to single-stranded oligonucleotides; and (ii) oligonucleotide 177–133M2 contains exactly the same point mutations as used by Karsenty and de Crombrugghe [12], which abolished IF-1 binding, whereas we saw no change in binding with chk-YB-1b due to these mutations, suggesting that our factor is different from IF-1. Another factor (BF COL1), isolated recently by de Crombrugghe's group [6], has also been shown to bind to the same polypyrimidine tract that chk-YB-1b recognizes. However, BF COL1 binds to double-stranded DNA. Other proteins known to bind within this stretch of the type-I procollagen promoters include SP1, Krox1 and proteins such as SPR2 [6]. Considering the fact that this region is essential for transcription from type-I procollagen promoters, it becomes very important to assess the relative contribution of each of these factors to collagen-gene transcription. Additionally, an investigation of interaction between these different factors would be worthwhile.

Type-I collagen is a heterotrimer formed from two α1(I) chains and one α2(I) chain. The transcriptional mechanisms which regulate the co-ordinate expression of the *α1* and *α2* genes are poorly understood. Although *α1* and *α2* genes are co-ordinately expressed, there are few obvious similarities between the two promoter sequences other than the pyrimidine/purine-rich sequences. The polypyrimidine/purine stretch seems to be very important for regulation of collagen genes, as it (i) maps to the DNaseI-sensitive sites in the transcriptionally active promoter [9]; (ii) is conserved among *α1* and *α2* promoters of type-I procollagen; and (iii) has been shown to contain target sequences for several trans-acting factors [6,11–13,25]. Herein, we have shown that chk-YB-1b binds the pyrimidine-rich regions in both *α1(I)* and *α2(I)* promoters from several species. Further, our results indicate that chk-YB-1b is involved in regulating the rat *α1(I)* promoter-driven transcription. Considering these results and the fact that chk-YB-1b is also known to be capable of binding the chicken *α2(I)* collagen promoter [25], we propose that chk-YB-1b may in part be responsible for the co-ordinate regulation of these two genes.

In summary, we have shown here that chk-YB-1b is involved in the regulation of *α1(I)* collagen gene expression. We have also shown the interaction of chk-YB-1 with similar regions within the *α1(I)* and *α2(I)* procollagen promoters of different vertebrates. Finally, our results raise the prospect for co-ordinate regulation of *α1(I)* and *α2(I)* collagen genes by this transcription factor.

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