Y-box proteins interact with the S1 nuclease-sensitive site in the chicken $\alpha 2(I)$ collagen gene promoter

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The sequence of the chicken $\alpha 2(I)$ collagen promoter from -712 to -85 , relative to exon 1, has been shown to be important for transcriptional activity. Within this region a pyrimidine/purine asymmetrical element at -200 bp forms an *in vitro* S1 nuclease-sensitive site. The pyrimidine-rich strand of this element interacts specifically with single-stranded DNA-binding proteins present in fibroblast nuclear extracts [Bayarsaihan and Lukens (1996) *Biochem. J.* **314**, 293–296]. To identify these proteins we performed expression screening of a chick embryo fibroblast cDNA library using a single-stranded polypyrimidine sequence derived from this element. One of the isolated clones was found to encode a member of the cold-shock gene family, either chicken

YB-1 or a highly homologous protein. This protein and a known chicken Y-box protein were both found to bind sequence-specifically to the pyrimidine-rich strand of the pyrimidine/purine asymmetrical element in the chicken $\alpha 2(I)$ collagen promoter. The binding mechanism of these proteins could be based on the formation of a non-canonical triplex DNA structure (H-DNA). Although members of this widespread and conserved protein family have been reported to modulate the expression of a number of genes, the findings reported here provide the first evidence for a possible role of cold-shock proteins in the regulation of type I collagen genes.

INTRODUCTION

The regulation of the genes encoding type I collagens is a highly co-ordinated event which is tightly regulated during development [1]. The induction of the type I collagen genes that occurs when chick embryo chondrocytes are cultured in the presence of 5-bromo-2'-deoxyuridine for 7–8 days provides a useful experimental system for studying this regulation [2,3]. Previous studies aimed at identifying the *cis*-acting elements that control the induction of transcription of the $\alpha 2(I)$ collagen gene in this system showed that the DNA region from -1600 to $+46$, relative to the transcription start site at $+1$, has promoter activity in transfected fibroblasts or bromodeoxyuridine-treated chondrocytes, but has little activity in untreated chondrocytes [4]. Deletion of the fragment from -712 to -85 causes a large decrease in promoter activity, and mobility shift assays suggested that the region from -283 to -85 may be responsible for the major complex formed between this fragment and fibroblast nuclear proteins [4]. Within this region a pyrimidine/purine asymmetrical sequence approx. 200 bp upstream from the transcription start site has been proposed to be an important regulatory element [5]. We have recently found that this polypyrimidine sequence is bound with high affinity by single-stranded-DNA-binding proteins that are present in nuclear extracts from chick embryo fibroblasts [6]. Several lines of evidence suggest that this region is likely to play a role in the regulation of this gene. First, the pyrimidine element is highly conserved between chicken and different mammalian species [5]. Secondly, a DNase I hypersensitive site maps to this region in the chromatin of cells with an active promoter, but is absent when the promoter is inactive [3,7]. Thirdly, several sequence- and tissue-specific DNA-binding proteins interact with this region ([6]; D. Bayarsaihan, B. Enkhmandakh and L. N. Lukens, unpublished work). Finally, this region when present in negatively

supercoiled plasmids contains S1 nuclease-sensitive sites, which indicates that this element can assume a non-B-DNA structure, such as H-DNA [8–10].

In order to find out more about the fibroblast nuclear proteins that interact with this pyrimidine-rich sequence in the $\alpha 2(I)$ collagen promoter, we used an expression cloning strategy to isolate cDNA clones for proteins that bind this sequence. We report here that one of the isolated clones encodes a chicken Y-box binding protein (chkYB-1) or a closely related protein [11]. This protein interacts with the single-stranded polypyrimidine sequence present in the $\alpha 2(I)$ collagen promoter with high affinity and in a sequence-specific manner. We have also tested a known member of the chicken YB-1 family, RSV-EF-I [12], and found that it shows identical DNA-binding behaviour. These proteins are members of the cold-shock protein family that is involved in the transcriptional regulation of many different genes [13–16]. There is also evidence that they can induce or stabilize a nuclease-sensitive state of DNA under physiological conditions [17]. The ability of the cold-shock proteins to bind the pyrimidine element within the S1 nuclease-sensitive site of the $\alpha 2(I)$ collagen promoter suggests that this interaction might modulate the expression of this gene by stabilizing an altered DNA structure.

MATERIALS AND METHODS

Oligonucleotides

Sequences of oligonucleotides used in the present work were as follows, with mutated nucleotides underlined: CT, TCTTCCCTCCCTTCTCCCTCCCTC; GA, GGGCGAGGGAGG-GAGGAAGGGAGGG; WT, double-stranded CT/GA oligonucleotide; ssM1, TCTTCCGTGGCTTCCTCCCTCCCTC; ssM2, TCTTCCCTCCCTTCCTCGGTCCTC; ssM3, TCTTCCGTGGCTTCCTCGGTCCTC; ssM4, TCTTCCCTC-

Abbreviations used: chkYB-1, chicken Y-box binding protein; IPTG, isopropyl β -D-thiogalactoside; DTT, dithiothreitol; β -gal, β -galactosidase.

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CCTAGGTCCTCCCTC; ssSp1, TTCAGACGTTCCCCG-CCCCTC; EA1, GATCCACATCATACCTAGCAG; CCAAT, double-stranded version of GGTCCTCTCTATTGGCC-GAGGGGC. Double-stranded oligonucleotides were prepared by annealing the complementary strands, and were purified from single-stranded oligonucleotides on a 16 % polyacrylamide gel. Single-stranded CT oligonucleotide was radiolabelled using T4 polynucleotide kinase (New England Biolabs) and [γ - 32 P]ATP (Amersham). Labelled nucleic acids were purified using a Microcon-3 spin column (Amicon).

cDNA library screening

A 10-day-old chicken embryo cDNA library in λ gt11 (Clontech) was screened according to the standard procedure [18]. Briefly, bacterial strain Y1090 was transfected with recombinant phage, plated and incubated at 42 °C for 3 h. The plates were overlaid with nitrocellulose filters pretreated in 10 mM isopropyl β -D-thiogalactoside (IPTG) and incubated at 37 °C for 6 h. The filters, containing about 600 000 phage, were incubated in binding buffer containing 25 mM Hepes, pH 8.0, 5 mM MgCl_2 , 50 mM NaCl and 1 mM dithiothreitol (DTT) supplemented with 5 % (w/v) non-fat dry milk for 1 h at 22 °C. Binding was carried out in the same buffer without milk, containing 10^5 c.p.m./ml 32 P-end-labelled single-stranded CT oligonucleotide. Filters were then washed in binding buffer for 4 $\times$ 10 min, dried and analysed by autoradiography. DNA inserts from positive clones were directly amplified from phage lysates by PCR with λ gt11 forward and reverse primers and then subcloned into pGEM-T vector (Promega). Plasmid DNA was purified with Qiagen columns and used for sequencing with Sequenase 2.0 (United States Biochemical). The sequences were subjected to homology searches using the Blast program from NCBI [19].

Production of β -galactosidase (β -gal) and His-tag fusion proteins

To produce β -gal fusion proteins, 50 000 plaques were plated in top agarose (Life Technologies) on 150 mm plates containing 0.4 mM IPTG. Plates were incubated at 42 °C for 3 h, and then at 37 °C for 16 h. The β -gal fusion proteins were eluted from the plates by placing 10 ml of elution buffer (20 mM Tris, pH 7.9, 1 % SDS, 10 % glycerol, 1 mM DTT, 0.1 mM EDTA and 25 nM PMSF) directly on the surface of the plate and incubating for 1 h at 22 °C with gentle agitation. The eluate was cleared by centrifugation and concentrated with a Microcon-100 filtration unit (Amicon). For production of the clone-CT-10-encoded protein (see the Results section) as a His-tagged fusion protein in a bacterial system, the *Eco*RI cDNA fragment from the recombinant phage was cloned into the *Eco*RI site of the pET-30c vector (Novagen). The plasmid was introduced into *Escherichia coli* strain BL21, and the transformed cells were cultured in LB broth to an absorbance of 0.6 unit, after which IPTG (1 mM) was added and the cultures were incubated for an additional 3 h at 37 °C. Total bacterial proteins were prepared and fusion proteins were purified using a nickel column according to Novagen protocols. Affinity-purified proteins were concentrated with a Microcon-30 filtration unit (Amicon). Protein concentration was assayed using the BCA kit (Pierce) with a BSA standard.

Southwestern and Western blots

The β -gal fusion proteins, produced by recombinant λ gt11 phage, were separated by SDS/PAGE on a 7.5 % gel and transferred to a nitrocellulose membrane [20], which was then blocked with 5 %

dry milk in binding buffer. The filter was hybridized in a final volume of 10 ml containing 200 000 c.p.m./ml radiolabelled probe for 16 h at 4 °C, and then washed several times in binding buffer. Autoradiography of the membrane was performed at -70 °C for 18–36 h. For the Western blot analysis, the filter was hybridized in a final volume of 10 ml containing anti- β -gal antibodies (Boehringer Mannheim) at 1:1000 dilution and then processed according to the Amersham ECL kit protocol.

Electrophoretic mobility shift and UV cross-linking assays

Protein–DNA complexes were identified by running samples on 5 % non-denaturing PAGE (29:1, acrylamide/bisacrylamide). The labelled oligonucleotide probe (30 000 c.p.m./ng) was incubated with protein samples (3–5 μ g of β -gal fusion protein or 0.1–1.0 μ g of affinity-purified protein) for 15 min at 22 °C in a reaction buffer containing 20 mM Tris/HCl, pH 7.9, 2 mM MgCl_2 , 50 mM KCl, 1 mM DTT, 8 % glycerol and 1 μ g of poly(dI-dC). In competition assays, the binding reaction was carried out in the presence of an excess amount, as indicated, of unlabelled competitor oligonucleotide for an additional 10 min prior to the addition of the radiolabelled probe. For UV cross-linking experiments the standard gel mobility-shift binding reactions were carried out with the β -gal fusion protein, and the samples were then irradiated on the surface of a UV trans-illuminator (UVP, model TM 20) at 4 °C at 254 nm for 60 min. The binding reaction was adjusted to a final concentration of 2 % SDS, 10 % glycerol, 0.1 M Tris, pH 6.8, 1 mM DTT and 0.02 % Bromophenol Blue with protein sample buffer, boiled for 5 min and analysed by 7.5 % SDS/PAGE. Gels were dried and analysed by autoradiography.

RESULTS

Screening of 6×10^5 plaques from a chick fibroblast cDNA library in λ gt11 with labelled CT oligonucleotide, which contains the pyrimidine-rich sequence present in the chicken $\alpha 2(\text{I})$ collagen promoter, TCTTCCCTCCCTTCCTCCCTCCCTC, yielded 24 positive plaques which, after two additional re-screenings, yielded 11 positive plaques. To examine binding specificity, one of them, designated CT-10, was then screened for its ability to bind the labelled CT probe in the presence of either excess unlabelled CT oligonucleotide or excess unlabelled mutant M3 oligonucleotide, which does not bind fibroblast nuclear factors [6]. An excess of the wild-type CT sequence competed for binding but an excess of M3 did not, indicating the sequence-specific nature of binding to this pyrimidine sequence (results not shown).

To evaluate further whether this binding activity was due to the fusion protein produced by the CT-10 lysogen, Western blots of extracts containing the CT-10 fusion protein prepared from λ lysogens were stained with anti- β -gal antibodies. The lysogenic extract contained a β -gal fusion protein of about 150 kDa (results not shown). When lysogen extracts containing λ gt11 without insert were treated similarly, no protein of this size was detected. To determine if this fusion protein could bind the pyrimidine element, a Southwestern blot was carried out. The membrane was probed with 32 P-labelled CT oligonucleotide, and only the protein product of approx. 150 kDa from the CT-10 lysogen extract showed binding activity (Figure 1).

Further evidence that the insert in the CT-10 phage encoded a DNA-binding protein was obtained by a UV cross-linking experiment (Figure 2). The labelled CT oligonucleotide was found to be cross-linked to a protein in the CT-10 lysogenic extract of the predicted size for the fusion protein (Figure 2, lane 2, arrowhead) and to another faster migrating protein, which is

Figure 1 Southwestern blot analysis shows that the CT-10 β -gal fusion protein binds the single-stranded CT sequence

The proteins in lambda lysogen extracts were separated by 7.5% SDS/PAGE and electroblotted to a nitrocellulose membrane. The filter was then probed with labelled CT oligonucleotide. The arrowhead indicates the CT-10 DNA-binding protein. Protein standard molecular masses ('Rainbow'; Amersham) are indicated on the left (in kDa).

Figure 2 UV cross-linking reveals binding of the CT-10 β -gal fusion protein to the CT sequence

The binding reaction with proteins from the lysogen extracts was performed in the presence of labelled CT oligonucleotide. The DNA-protein complex was then UV-irradiated (254 nm) for 60 min and separated on 7.5% SDS/PAGE followed by autoradiography. The arrowhead indicates the adduct between the CT probe and the undegraded CT-10 fusion protein. The labelled CT oligonucleotide was found to be cross-linked to a protein in the CT-10 lysogenic extract of the predicted size for the fusion protein (lane 2, arrowhead) and to another, faster migrating protein, which is a proteolytic product of the fusion protein. No discrete cross-linked band was seen in the control lane that contained the lysogenic extract from λ gt11 containing an ovalbumin cDNA insert (lane 1). Protein standards ('Rainbow'; Amersham) are indicated on the left (in kDa).

believed to be a proteolytic product of the fusion protein. No discrete cross-linked band was seen in the control lane that contained the lysogenic extract from λ gt11 containing an ovalbumin cDNA insert (Figure 2, lane 1).

The 1.1 kb cDNA insert in the CT-10 phage was partially sequenced from both ends, after PCR amplification and subcloning. Computer analysis revealed that the sequenced portions of clone CT-10 are identical to the cDNA sequence encoding chkYB-1 [11], from bp 301 to 508 and from bp 925 to 1121 inclusive. The protein coding sequence of the chkYB-1 cDNA goes from bp 134 to 1099 inclusive, with the sequence from bp 305 to 502 encoding the conserved cold-shock domain [11]. The first sequence from CT-10 corresponds, therefore, to 208 bp of the cold-shock domain and the second sequence

Figure 3 Gel mobility-shift assays of the binding of the affinity-purified His-tagged CT-10 protein to the single-stranded CT oligonucleotide

All lanes contained the fusion protein and the radiolabelled CT probe. Lane 1 contained no competitor. A molar excess, as indicated, of the unlabelled single-stranded or double-stranded oligonucleotides was added to lanes 2–11.

Figure 4 Southwestern blot analysis shows that the affinity-purified (maltose-binding protein)-RSV-EF-1 fusion protein binds the single-stranded CT sequence

Purified fusion proteins were separated by 7.5% SDS/PAGE and electroblotted to a nitrocellulose membrane. The filter was probed with labelled single-stranded CT oligonucleotide. The arrowhead indicates the major undegraded fusion protein that binds to the CT sequence. Protein standards ('Rainbow'; Amersham) are indicated on the left (in kDa).

corresponds to the last 175 bp of the C-terminal coding region plus 22 bp of the 3' untranslated sequence [11].

Y-box binding proteins have been previously reported to bind to both double- and single-stranded DNA in a sequence-specific manner (reviewed in [21]). To examine the binding activity of the protein encoded by clone CT-10, the His-tag fusion protein was purified on an affinity column and used in mobility-shift experiments (Figure 3). Various oligonucleotides were used as competitors in binding reactions to determine the recognition target of the CT-10 protein. The results showed that the purified protein bound preferentially to the single-stranded CT oligonucleotide that contains the polypyrimidine sequence present in the $\alpha 2(I)$ collagen promoter. A 20-fold excess of this unlabelled sequence was enough to compete away the binding of the 32 P-labelled CT oligonucleotide (Figure 3, lane 2), whereas a 20-fold excess of the mutant sequences M1, M2 or M3 did not compete, nor did the inverted double-stranded CCAAT motif or the single-stranded oligonucleotides Sp1 and EA-1 (Figure 3, lanes 4 and 6–11). The double-stranded CT sequence (WT) partially

Figure 5 Gel mobility-shift assays of the binding of the affinity-purified (maltose-binding protein)-RSV-EF-I fusion protein to the single-stranded CT oligonucleotide

All lanes contained the fusion protein and probe. (A) Lane 1 contained no competitor; lanes 2–4 contained a molar excess of the unlabelled single-stranded CT competitor, as indicated; lanes 5–7 contained a molar excess of the unlabelled double-stranded CCAAT competitor, as indicated. (B) Lanes 2–5 contained increasing amounts of the unlabelled double-stranded wild-type CT competitor, as indicated. Lane 1 contained no competitor.

competed (lane 3) and the single-stranded mutant sequence ssM4, previously shown to retain the ability to be bound by fibroblast nuclear factors [6], competed as well as did the wild-type CT sequence (lane 5). These binding specificities are similar to those previously observed with the factor or factors present in nuclear extracts from fibroblasts [6].

To obtain additional evidence that cold-shock proteins bind to the polypyrimidine sequence in the $\alpha 2(I)$ collagen promoter, we tested whether the binding behaviour of a recently described member of the chick YB-1 family, RSV-EF-I [12], is similar to that of the CT-10 protein. RSV-EF-I is believed, on the basis of an overall 98 % sequence identity, to be identical with chkYB-I [12]. An affinity-purified fusion protein of approx. 80 kDa between maltose-binding protein and RSV-EF-I (a gift from Dr. R.V. Guntaka, University of Missouri, Columbia, MS, U.S.A.) was shown by Southwestern blot analysis to bind the single-stranded CT oligonucleotide (Figure 4, arrowhead). The faster-migrating species were due to partial proteolysis. This protein resembles the CT-10 protein in its binding specificity, showing the strongest affinity for the single-stranded CT oligonucleotide (Figure 5A, lanes 2–4) and less affinity for the double-stranded CT sequence (Figure 5B). It had even less affinity for the inverted CCAAT sequence (Figure 5A, lanes 5–7), although a 300-fold excess of this latter sequence was able to compete away the binding of the CT oligonucleotide to either the RSV-EF-I or the CT-10 purified fusion protein (results not shown). The stronger affinity of chicken Y-box binding proteins for single-stranded pyrimidine sequences, as compared with a double-stranded sequence containing an inverted CCAAT motif, has been noted by others [22–24], although this latter motif was present in the original DNA sequence that was designated the 'Y-box' [21].

These DNA-binding studies show that the Y-box protein encoded by clone CT-10, and the previously characterized Y-box protein RSV-EF-I, bind specifically and with high affinity to single-stranded DNA containing the pyrimidine-rich sequence present in the $\alpha 2(I)$ collagen promoter.

DISCUSSION

Co-ordinated expression of the genes encoding type I collagens is believed to be determined by a series of *cis*-acting elements located upstream of the transcriptional start site [25,26]. Within

this region of the chicken $\alpha 2(I)$ collagen gene there is a pyrimidine/purine asymmetrical element which is very well conserved among vertebrates [5]. We have recently found that this element contains target sequences for several specific DNA-binding proteins present in nuclear extracts from embryonic chick fibroblasts and chondrocytes [6]. Some of these nuclear proteins have a very high affinity for the pyrimidine-rich single-stranded sequence, and are expressed only in fibroblasts [6].

Here we report that one of the fibroblast nuclear proteins that binds with high affinity to this single-stranded pyrimidine sequence is identical or closely related to the previously cloned chkYB-1 protein [11,12]. This protein belongs to the Y-box family of proteins, which share a highly conserved DNA-binding region, known as the cold-shock domain from its similarity to bacterial cold-shock proteins (reviewed in [21,27]). These proteins have recently been the subject of intense interest, because they are exceptionally well conserved between species, yet they have been implicated in a wide variety of different functions in different physiological situations [28–30]. Members of this family of proteins can interact not only with double-stranded DNA but also with RNA and single-stranded DNA [17,22,23,31,32]. In particular, some cold-shock proteins bind with high affinity to many, but not all, pyrimidine-rich single-stranded DNA sequences [22,23]. The basis for this preference for particular pyrimidine sequences is not yet known.

The polypyrimidine site in the $\alpha 2(I)$ collagen promoter shows a striking resemblance in two respects to the polypyrimidine sites in two other promoters, those for the human *c-myc* and γ -globin genes. First, all three DNA regions become sensitive to cleavage by S1 nuclease *in vitro* when present in a negatively supercoiled plasmid [8,9], apparently due to the formation of triplex H-DNA [10,23,33]. Secondly, all three regions bind nuclear factors (NSEP and dbpB respectively in the case of the *c-myc* and γ -globin promoters) that are, like the chicken Y-box protein reported here, YB-1-type proteins [22,23,34,35]. In the case of the *c-myc* and γ -globin promoters, evidence has been obtained that suggests that these proteins may recognize an altered DNA secondary structure of the polypyrimidine sequence *in vivo* [23,36]. For γ -globin, it has been shown that BP-8 binds much more avidly to the double-stranded polypyrimidine sequence under conditions that are known to favour H-DNA structures [23]. In the *c-myc* promoter, mutations that decrease the stability of the H-DNA

conformation also show decreased activity in transfection experiments [36].

The YB-1 protein that we have isolated, both as a β -gal fusion protein and as an affinity-purified His-tagged protein, binds the pyrimidine sequence of the $\alpha 2(I)$ collagen promoter with high affinity. A previously identified member of the chicken cold-shock protein family, RSV-EF-I, which is believed to be identical to chkYB-1 [12], was also found to bind with similar affinity to this sequence. Members of this family have been found to either activate or repress transcription [13–16]. This specific interaction of cold-shock proteins with the pyrimidine element may, therefore, contribute to the regulation of expression of the $\alpha 2(I)$ collagen gene.

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