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An Activator Protein-1 (AP-1) Response Element on Pro α_1(I) Collagen Gene is Necessary for Thyroid Hormone-Induced Inhibition of Promoter Activity in Cardiac Fibroblasts

Hyeon-Woo Lee, Laura E. Klein, Jonathan Raser and Mahboubeh Eghbali-Webb

Department of Anesthesiology, Yale University School of Medicine, New Haven, CT 06510, USA

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H.-W. Lee, L. E. Klein, J. Raser and M. Eghbali-Webb. An Activator Protein-1 (AP-1) Response Element on Pro α_1(I) Collagen Gene is Necessary for Thyroid Hormone-Induced Inhibition of Promoter Activity in Cardiac Fibroblasts. *Journal of Molecular and Cellular Cardiology* (1998) **30**, 2495–2506. Thyroid hormone-induced ventricular hypertrophy is characterized by the absence of fibrosis. Previously, we demonstrated that thyroid hormone inhibits collagen type I gene expression in the myocardium and in cardiac fibroblasts. We also demonstrated that thyroid hormones act as inhibitor of pro α_1(I) collagen promoter activity. In this study we determined the sequences on pro α_1(I) collagen gene and transcription factors in cardiac fibroblasts involved in the inhibitory effect of 3,3′,5-triiodothyronine (T_3). Transient transfection of cells with chloramphenicol acetyl transferase (CAT)-linked deletion mutants of pro α_1(I) collagen promoter demonstrated that the inhibitory effect of T_3 is transmitted via proximal sequences ($-225/+115$). Gel shift analysis using [^{32}P]-labeled $-225/+115$ gene fragment and nuclear proteins of cardiac fibroblasts showed T_3-induced DNA binding by two proteins. Analysis of non-overlapping restriction sub-fragments by gel shift along with supershift analysis with antibodies to types α and β thyroid hormone receptors identified the lower molecular weight DNA-binding protein as β receptor and confirmed that the T_3-induced protein-DNA binding sites are located at $-15/+115$. Selective base mutation (C in place of G at $+93$ and G in place of C at $+97$) in the activator protein-1 (AP-1) core binding motif ($+92/+97$) abolished the higher molecular weight T_3-induced DNA-protein complex obtained with [^{32}P]-labeled wild type sequences ($-225/+115$). Additional gel shift analyses using an oligonucleotide containing the AP-1 core binding motif, as an unlabeled competitor and as [^{32}P]-labeled probe, confirmed the T_3-induced protein binding to an AP-1 site. Transient transfection with CAT-linked $-225/+115$ sequences in which the AP-1 site was mutated abolished the T_3-induced inhibition of CAT activity. Together, these findings identify sequences necessary for T_3-induced inhibition of collagen type I promoter to which thyroid hormone receptor type β and protein(s) with affinity for AP-1 element bind. They also demonstrate that the AP-1 response element located on these sequences is necessary for T_3-induced inhibition of pro α_1(I) collagen promoter activity. These data identify molecular mechanisms involved in thyroid hormon-induced inhibition of collagen expression in the heart.

Key Words: Myocardial hypertrophy; Thyroid hormone; Transcription; Hyperthyroidism; Gene regulation; AP-1.

Introduction

Of all the known forms of pathological ventricular hypertrophy, thyroid hormone-induced hypertrophy is unique in that it lacks fibrosis and is completely reversible, and as such closely resembles

Please address all correspondence to: Mahboubeh Eghbali-Webb, Department of Anesthesiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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physiologic hypertrophy due to exercise. Previously, we established, using rats with hyperthyroidism (Yao and Eghbali, 1992a) and thyroidectomized rats (Klein *et al.*, 1996) that circulating thyroid hormones down-regulate collagen type I biosynthesis in the heart. We also showed that thyroid hormone-treatment of tight skin mouse, a genetic model of cardiac fibrosis, led to normalization of an otherwise exaggerated collagen type I gene expression in ventricular tissue (Yao and Eghbali, 1992b). Our *in vitro* studies with thyroid hormone-treated cardiac fibroblasts, the cellular source of collagen type I in the heart, showed thyroid hormone-induced decreased levels of collagen type I mRNA with only moderate changes in mRNA stability (Yao and Eghbali, 1992a). Transient transfection of cardiac fibroblasts with chimeric DNA containing 3.6 kb promoter sequences of pro α_1(I) collagen linked to the reporter gene, chloramphenicol acetyl transferase (CAT), showed a significant decrease in the activity of collagen type I promoter in thyroid hormone-treated cells (Yao and Eghbali, 1992a).

Thyroid hormones act through the intracellular receptor types α and β which are the product of c-*erb*A protooncogene and belong to the superfamily of ligand-activated transcription factors. These receptors by binding to thyroid hormone response elements on the target promoters can act as activator or repressor of transcription (Umesono *et al.*, 1991). The 5′ flanking regions of collagen type I promoter contain *cis* regulatory domains that control the constitutive and hormone- and growth factor-regulated expression of pro α_1(I) collagen gene. Examples include enhanced transcription of collagen type I by transforming growth factor-beta (TGF-β_1) (Rossi *et al.*, 1988), its decrease by 1,25-dihydroxyvitamin D (Lichtler *et al.*, 1989) its down-regulation in transformed fibroblasts (Schmidt *et al.*, 1985), and decreased α_1 (I) collagen promoter activity by parathyroid hormone (Kream *et al.*, 1993). Although previously we have reported the thyroid hormone-induced inhibition of pro α_1(I) collagen promoter (Yao and Eghbali, 1992a), the thyroid hormone-sensitive *cis*-acting domains on this promoter have not been yet identified.

The AP-1 (activator protein factor 1 in HeLa cells) transcription factor interacts with DNA sequences (AP-1 response element) on target promoters as c-jun homodimer or c-jun/c-fos heterodimer and induces positive or negative regulation of gene expression for diverse proteins involved in multiple aspects of cell growth, development and differentiation. Previously, we demonstrated thyroid hormone-induced expression of c-fos and c-jun mRNAs in cardiac fibroblasts and showed that similar to thyroid hormones, phorbol myristate acetate (PMA) which induces the expression of c-fos and c-jun mRNAs, causes down regulation of collagen type I gene expression in cardiac fibroblasts (Eghbali *et al.*, 1991). In this study, we tested the hypothesis that the observed inhibitory effect of thyroid hormones on pro α_1(I) collagen promoter is mediated via interaction of T_3-induced nuclear proteins with promoter sequences and investigated the possibility of involvement of an AP-1 response element on pro α_1(I) collagen promoter in T_3-induced inhibition of promoter activity.

Materials and Methods

Cell culture and hormone treatment

Cultured cardiac fibroblasts were prepared as previously described (Yao and Eghbali, 1992a). Adult male (White New Zealand) rabbits were anaesthetized, their hearts were excised, left ventricular tissue was minced, and washed in phosphate buffered saline (PBS). The tissue was then subjected to digestion at 35°C by a mixture of 0.1% trypsin and 100 U/ml collagenase (type IV, Sigma) in a 25 ml glass Erlenmeyer flask, for 10 min by constant shaking. Isolated cells were pelleted at the end of each 10 min digestion period. Cells from the third to ninth digestion were plated on 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) and incubated for 2 h at 37°C in a humidified incubator with 10% CO_2. At the end of this period, the unattached cells were discarded, and attached cells were grown in DMEM + 10% FBS. The nature of cells was determined by immunofluorescence staining with anti-human factor VIII, anti-desmin and anti-vimentin for identification of endothelial cells, muscle cells and fibroblasts, respectively, as previously described (Eghbali *et al.*, 1989). In passage 1, only 1–2% of cells stained positively with anti-desmin and anti-factor VIII. All cells were stained positively with anti-vimentin antibody (Eghbali *et al.*, 1989). In this study cells from passages six to eight were used. At this stage all cells stain negative for desmin or factor VIII and maintain their fibroblastic characteristics. For treatment with thyroid hormone, confluent cells were serum-deprived for 24 h and then incubated with T_3 (10 nM) or an equivalent volume of the vehicle (0.1 N NaOH) for 2–24 h.

Preparation of nuclear extract

Cell nuclei were prepared and nuclear proteins extracted according to the method of Shapiro *et al.* (Shapiro *et al.*, 1988). Briefly, cells were harvested and subjected to hypotonic treatment (10 mM, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 10 mM KCl) and then broken with three strokes from a tight fitting dounce. Immediately thereafter 0.1 volume sucrose restore buffer [9 volumes of 75% RNase-free sucrose (w/v) + 1 volume of 10 × salts (0.5 M Hepes, pH 7.9, 7.5 mM spermidine, 1.5 mM spermine, 0.1 M KCl, 2 mM EDTA, 10 mM dithiothreitol)]. The nuclei were pelleted at 16 000 × **g** at 4°C and resuspended in a buffer containing 1 volume saturated ammonium sulfate and 9 volumes of a stock containing 20 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 25% glycerol. The mixture was rocked 30 min at 4°C, centrifuged at 150 000 × **g** for 90 min at 2°C and the concentration of ammonium sulfate in supernatant was adjusted to 0.33 g/ml. The mixture was rocked at 4°C (20 min) and pelleted at 85 000 × **g** (20 min at 4°C) and redissolved in nuclear dialysis buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol). Ultrafiltration, via multiple cycles of centrifugation through an Amersham Microcon-3 microconcentrator and redilution with nuclear dialysis buffer was followed by concentration of the final extract. Aliquots of the extract were used for determination of protein concentration by use of a BCA™ Protein Assay Kit (Pierce, Rokford, IL, USA) according to the manufacturers guidelines.

DNA probes

Restriction fragments

The DNA fragments used as probes were derived from construct Col Cat 3.6 (generous gift of Dr David Rowe, University of Connecticut) (Lichtler *et al.*, 1989) This construct had been originally produced by ligation of a 3635-bp fragment of rat pro α_1(I) collagen promoter which contains 5′-untranslated region, the presumptive mRNA start site and an additional 115 bp intronic sequences, into the *Xba*I site of a CAT plasmid cloned into pUC12 (Kream *et al.*, 1993). In this study, the construct Col Cat 3.6 was digested with *Xba*I and the 3635-bp promoter fragment was isolated from the vector DNA by electrophoresis in a 1% agarose gel. A 340-bp *Bgl*II-*Xba*I (−225/+115) was isolated by restriction digestion. In addition, two non-overlapping *Apa*I restriction digest subfragments (−225/−15 and −15/+115) of the 340-bp fragment were obtained.

Oligonucleotides

An oligonucleotide containing the core binding motif of AP-1, called AP-1 consensus oligonucleotide was purchased from Promega (Madison, WI). Both strands of the 31 bp (5′-AGCTTA-GGACGTTGGGGTTAGGGGAGGACAGTGGCA-3′) malic enzyme thyroid hormone response element (Desvergene *et al.*, 1991) were synthesized (at the W.M. Keck Biotechnology Resource Laboratory at Yale School of Medicine) on an Applied Biosystems Model 380B DNA synthesizer (Applied Biosystems, Foster City, CA, USA) and annealed as described by Kadonaga and Tjian (1986). These sequences in the 5′-flanking region of the malic enzyme gene confer T_3-inducibility of transcription to malic enzyme promoter, as well as to a heterologous promoter. Repression of transcription seen in the absence of T_3 also correlated with receptor binding to this region (Desvergene *et al.*, 1991).

Polymerase chain reaction

A fragment of pro α_1(I) collagen promoter containing nucleotides spanning −225/+115 sequences (wild type) and its mutant in which the AP-1 response element was mutated were synthesized in large quantities [3.6 kb pro α_1(I) collagen promoter was used as template] by polymerase chain reaction (PCR) using a Perkin-Elmer Cetus DNA amplification kit (Gene Amp RNA PCR kit) according to the manufacturer's guidelines. For wild type promoter oligonucleotides complementary to −225/−217 and +96/+115 sequences were used as sense and anti-sense primers, respectively. For mutated promoter an oligonucleotide complementary to −225/−217 was used as the sense primer and an oligonucleotide complementary to +85/+115 in which a C at position +93 was substituted by a G and a G at position +97 was substituted by a C was used as the anti-sense primer. Sense and anti-sense primers contained a *Hind*III site at their 3′ ends which enabled the subsequent subcloning of the PCR-generated fragments into the *Hind*III site of a reporter gene-containing plasmid as described below. In addition, a 119-bp promoter fragment spanning −28/+91 (lacking AP-1 element) and a 146-bp fragment spanning −28/+115 sequences (containing AP-1 element) were

synthesized by using an oligonucleotide complementary to sequences −28/−6 as the sense primer for both and oligonucleotides complementary to +72/+91 and +96/+115 sequences as antisense primers for 119-bp and 146-bp, respectively. These DNA fragments were used as competitor DNA or labelled probe in gel shift analysis. PCR was performed with 30 cycles (denaturing at 95°C, 1 min; annealing at 60/57°C, 1 min; extension at 72°C, 1 min and 30 s) in a Perkin-Elmer Cetus thymocycler. Aliquots of the PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining for size verification. Sequence verification was achieved by DNA sequencing using AmpliTaq FS DNA polymerase and fluorescent-dideoxy terminators (Perkin Elmer) in a cycle sequencing method and electrophoresis of the resultant DNA fragments using an automated Applied Biosystems 377 DNA sequencer (at the W. M. Keck Biotechnology Resource Laboratory at Yale School of Medicine).

DNA constructs

Chimeric DNA containing −900/+116 (PG100) and −222/+116 (PG60) sequences of pro α_1(I) collagen promoter ligated to the *Hind*III site of pAZ1003 plasmid in front of the CAT reporter gene described previously (Karsenty and de Crombrugghe, 1990) and were a generous gift of Dr Benoit de Crumbrugghe (University of Texas). In addition, a PCR-generated −238/+115 fragment in which the AP-1 site was mutated and with *Hind*III sites at both ends, was digested with *Hind*III and introduced into the *Hind*III site of pAZ1003. The orientation of the insert in the construct was verified by DNA sequencing as described above. Expression plasmid, pSV β-galactosidase, containing the β-galactosidase gene under the control of SV40 early promoter and enhancer as well as a SV40 promoter-CAT construct (pCAT™-promoter plasmid) were purchased from Promega (Madison, WI, USA).

Gel mobility–shift assay

Binding reactions were performed as described in Karsenty and de Crombrugghe (1990). Briefly, 5 fmol [^{32}P]-end-labeled DNA were incubated with 5–10 μg nuclear protein in a final solution consisting of 20 mM NaOH-Hepes, pH 7.9, 20% glycerol, 0.05 M KCl, 0.05 mM ZnCl dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 4 μg poly dI–dC for 60 min at room temperature. In some reactions molar excesses of competitor DNAs were added 60 min prior to the addition of the labeled DNA. In super shift reactions, polyclonal antibodies to α and β subtypes of thyroid hormone receptor (Bioaffinity Reagents) were incubated with the nuclear extract for 60 min prior to the addition of the labeled DNA. Non-immune rabbit serum was used as negative control. The products of binding were electrophoresed on nondenaturing (3.5–4.5%) polyacrylamide gels in Tris-Borate, pH 7.9 buffer. Gels were dried and exposed to Hyperfilm-MP™ (Amersham) at −80°C.

Transient transfection of cardiac fibroblasts

Cells were seeded at a density of 2.5×10^5 cells/60 mm dish, 16 h prior to transfection. Six micrograms of CAT constructs were co-transfected with 6 μg pSV β-galactosidase (as an internal standard for normalization of the results for transfection efficiency). DNA was transfected with 15 μg lipofectin (BRL, Gaitersburg, MD) for 8 h according to the manufacturer's guidelines. DNA-liposome complex was then removed and cells were grown in DMEM in the presence of 10% FBS for 12 h and then incubated in serum-free medium in the presence of 10 nM T_3 for 24 h. The effect of T_3 on the activity of SV40 promoter was monitored by transfection of SV40 promoter-CAT construct into cardiac fibroblasts.

Determination of promoter activity

The activity of pro α_1(I) collagen promoter was determined by measuring CAT activity according to Gorman *et al.* (1982). Multiple experiments were performed on each group (T_3-treated and vehicle-treated control cells). In each assay aliquots of cell lysate from T_3-treated and control cells containing equal amount of protein were processed for CAT activity. To normalize the results for transfection efficiency, matching aliquots from each sample were used for measurement of β-galactosidase activity, by the use of β-galactosidase enzyme assay system (Promega Co., Madison, WI, USA), according to the manufacturer's guidelines. To quantify CAT activity, the density of autoradiographic spots corresponding to acetylated [^{14}C]chloramphenicol and untransformed [^{14}C]chloramphenicol, was measured by densitometry scanning (Hoefer) of the autoradiographs. CAT activity was calculated as the percentage of acetylated [^{14}C]chloramphenicol in a

1 h reaction and the values were normalized to β-galactosidase activity. The effect of thyroid hormone on basic CAT expression was determined by using cytoplasmic extracts obtained from control and T_3-treated cardiac fibroblast harboring a pCAT™-promoter plasmid. Data obtained from T_3-treated groups were compared with those from control groups using the t statistics (GraphPad InPlot; GraphPad Software, San Diego, CA, USA).

Results

Previously, we demonstrated the T_3-induced inhibition of CAT expression under the control of a 3.6 kb (-3.5 kb/$+115$) fragment of pro α_1(I) collagen promoter (Yao and Eghbali, 1992a). In the present study, the minimal sequences on pro α_1(I) collagen promoter that are necessary for the T_3-induced inhibition of promoter activity in cardiac fibroblasts were determined by transient transfection of the cells with CAT constructs containing deletion mutants of pro α_1(I) collagen promoter, i.e., plamids containing $-900/+116$ and $-222/+116$ sequences of pro α_1(I) collagen (Karsenty and de Crombrugghe, 1990). The results of CAT assay showed that $-222/+116$ sequences of pro α_1(I) collagen promoter were able to confer T_3-induced inhibition of CAT expression and indicated the involvement of proximal sequences in thyroid hormone-induced inhibition of promoter activity (Fig. 1).

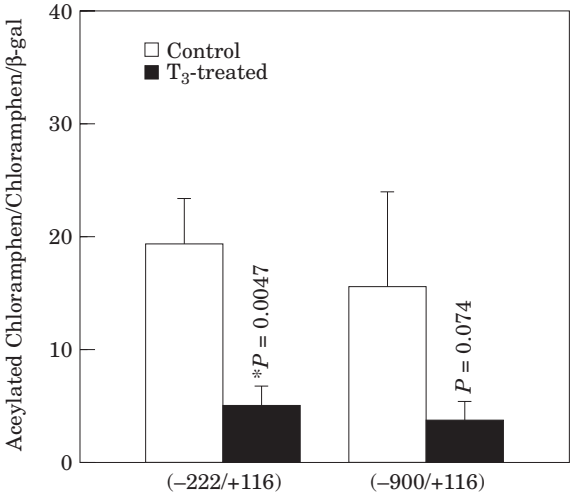

Figure 1 Effect of thyroid hormone on CAT expression under the control of collagen type I promoter in cardiac fibroblasts. Cardiac fibroblasts were transiently transfected with plasmids containing CAT-linked deletion sequences ($-900/+116$ and $-222/+116$) of pro α_1(I) collagen promoter. The plasmid pSV β-galactosidase was used as an internal standard for transfection efficiency. The effect of thyroid hormone (10 nM, 24 h) on expression of CAT in cytoplasmic extracts of fibroblasts harboring these constructs was determined as described in Methods. Thyroid hormone did not change basic CAT expression in cardiac fibroblasts harboring a pCAT™-promoter plasmid (data not shown). t statistics, $n=3$.

Thyroid hormone-induced DNA-protein binding

To determine the T_3-induced interaction of nuclear proteins in cardiac fibroblasts with proximal sequences of the promoter, we used a [^{32}P]-end-labeled 340-bp ($-225/+115$) restriction fragment of pro α_1(I) collagen promoter and nuclear proteins of cardiac fibroblasts in gel mobility shift analysis (a diagram of restriction digestions used in this study is provided in Fig. 2). The shift in the mobility of labeled DNA demonstrated two protein-DNA complexes (Fig. 3). Unlike the lower molecular weight DNA–protein complex, the higher molecular weight complex was not readily detectable with nuclear proteins of control cells and its detection with nuclear proteins of T_3-treated cells required higher concentrations of purified nuclear proteins (Fig. 3).

Furthermore, the kinetics of detection for the two protein-DNA complexes were different in that, the higher molecular weight complex was readily detectable with nuclear proteins obtained after 3–4 h of T_3-treatment and its concentration was reduced after longer periods up to 24 h (Fig. 3). The detection of lower molecular weight complex, on the other hand, required longer (24 h) duration of hormone treatment (Fig. 3). In competition studies, molar excess of an oligonucleotide containing the T_3 response element on malic enzyme gene specifically and in a dose-dependent manner, reduced the lower molecular weight protein-DNA complex while the higher molecular weight complex remained unchanged (Fig. 3).

To determine the location of protein-binding on the promoter more precisely, we used two non-overlapping subfragments ($-15/+115$ and $-225/-15$), resulted by *Apa*I digestion of the 240-bp fragment, in gel shift analysis. Of the two subfragments, the 130-bp, 3′ most subfragment ($-15/+115$) produced retardation signals similar to that observed with 340-bp fragment. The lower molecular weight signal was specifically competed with molar excess of double-stranded oligonucleotide corresponding to the T_3-response element on malic enzyme gene (Fig. 4). Super shift experiments with antibodies to type β thyroid hormone receptor showed a shift in the mobility of the 130-bp DNA–protein complex suggesting the

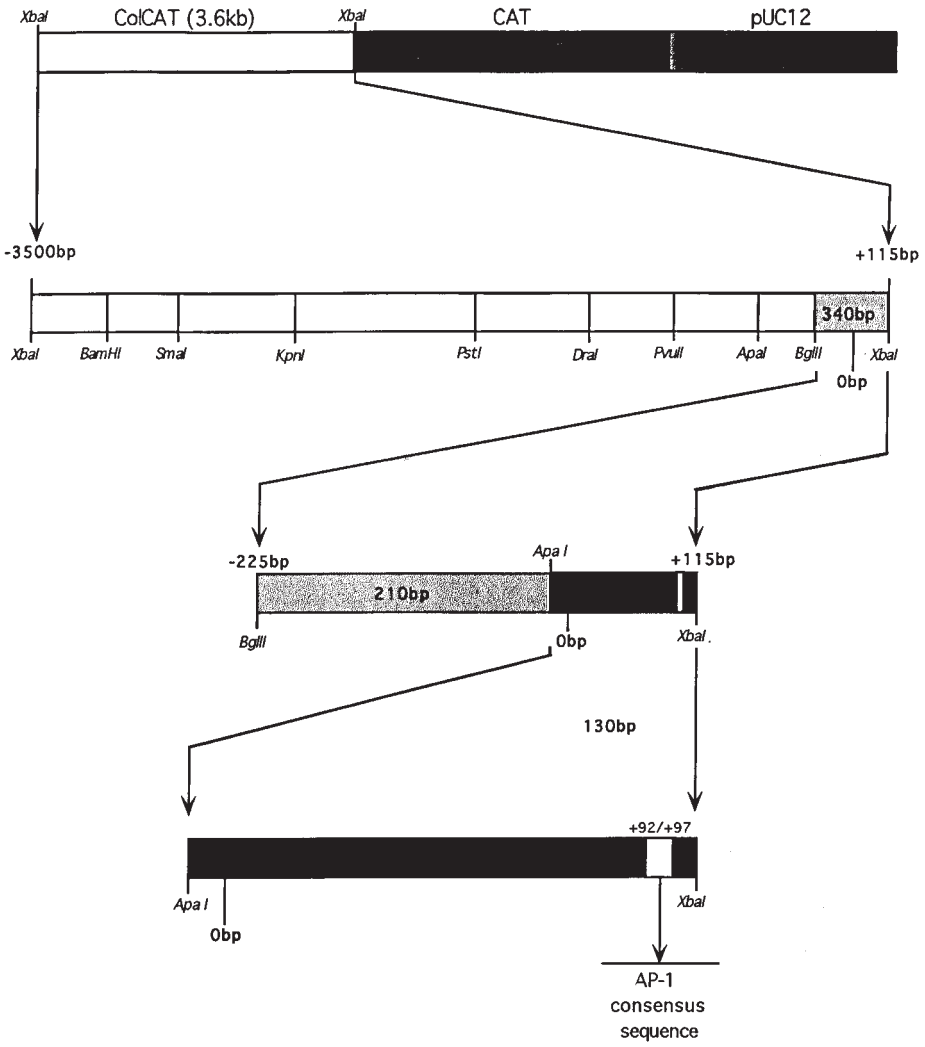

Figure 2 Schematic representation of enzymatic digestion of pro α_1(I) collagen promoter. The construct Col Cat 3.6 (Lichtler *et al.*, 1989) which had been originally produced by ligation of a 3635-bp fragment of rat pro α_1(I) collagen promoter which contains 5′-untranslated region, the presumptive mRNA start site and an additional 115 bp intronic sequences, into the *Xba*I site of a CAT plasmid cloned into pUC12 (Kream *et al.*, 1993) was digested with *Xba*I. The resulting 3635-bp promoter fragment was isolated from the vector DNA by electrophoresis in a 1% agarose gel. A 340-bp *Bgl*II-*Xba*I ($-225/+115$) was isolated by restriction digestion. In addition, two non-overlapping *Apa*I restriction digest subfragments ($-225/-15$ and $-15/+115$) of the 340-bp fragment were obtained.

involvement of β receptor in observed DNA-binding (Fig. 4). This effect was not observed with an antibody to type α receptor (Fig. 4). The 5′ most 210-bp ($-225/-15$) subfragment, did not produce retardation signals with nuclear proteins (Fig. 4).

Based on our previous studies which showed thyroid hormone-induced expression of mRNAs for c-fos and c-jun, in cardiac fibroblasts (Yao and Eghbali, 1992a) and the results of sequence analysis of the pro α_1(I) collagen promoter which showed that in addition to several upstream locations ($-2254/-2246$, $-1143/-1139$, $-515/-511$), an AP-1 response element is also located at $+92/+97$ (Fig. 5), we hypothesized that this site is involved in T_3-induced protein-binding to $-225/+115$ sequences of collagen type I promoter. To test this hypothesis, we used PCR to introduce a double-base mutation in the AP-1 element (a C at position $+93$ was substituted by a G and a G at position $+97$ was substituted by a C) on the 340-bp ($-224/+115$) promoter fragment. The results of gel shift analysis using the [^{32}P]-labeled mutated 340-bp showed the absence of higher molecular weight DNA-protein complex which was seen with the [^{32}P]-labeled wild type 340-bp (Fig. 6). This DNA-protein complex was also absent with [^{32}P]-labeled wild type 340-bp in the presence of molar excess of an unlabeled

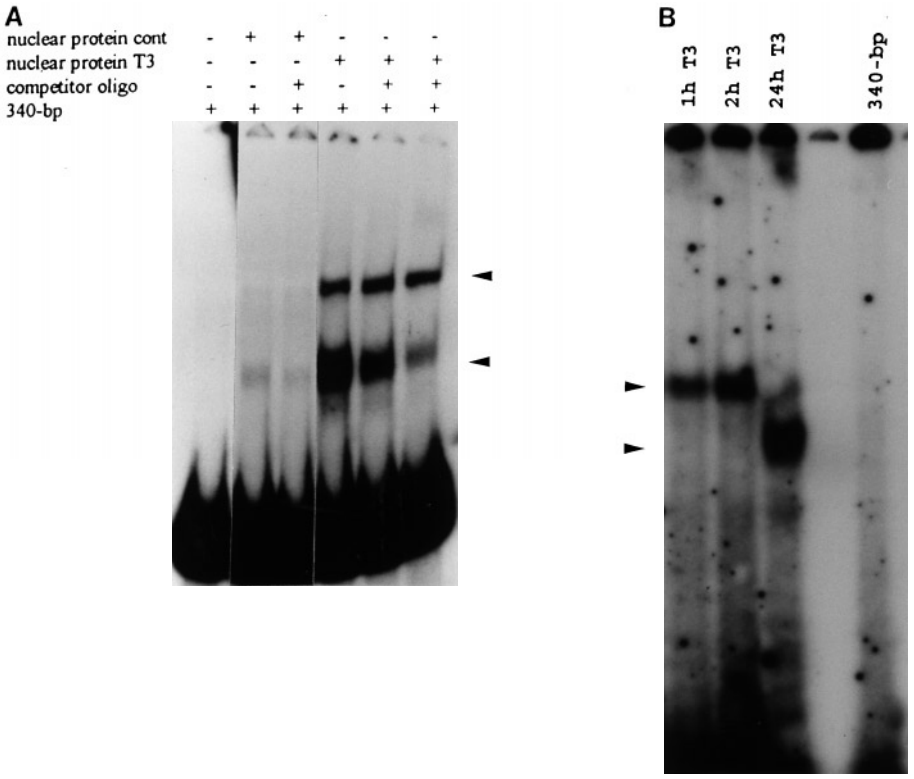

Figure 3 Gel mobility shift analysis of A 340-bp *Bgl*II-*Xba*I restriction fragment (−225/+115) of pro α_1(I) collagen promoter and nuclear proteins isolated from cardiac fibroblasts. In panel A nuclear proteins (10 μg) were prepared from T_3-treated (10 nM, 24 h) and control cells as described in Methods. A [γ-^{32}P]labeled (5 fmol) 340-bp (−225/+115) fragment of promoter was used as labeled DNA in gel mobility shift as described in Methods. Molar excess (10, 50) of double-stranded oligonucleotide corresponding to the T_3-response element on malic enzyme gene was used as unlabeled competitor. This oligonucleotide was pre-incubated with nuclear proteins followed by incubation with the labeled DNA and electrophoresis on a non-denaturing 3.5% polyacrylamide gel. Panel B shows the time course of T3-induced expression of high and low molecular weight DNA-binding proteins. These autoradiographs are representatives of three independent experiments.

competitor oligonucleotide containing the AP-1 response element (Fig. 6), suggesting that in addition to T_3 receptor type β, protein(s) with binding affinity for AP-1 response element are involved in T_3-induced protein-DNA binding in cardiac fibroblasts. The lower molecular weight DNA–protein complex, on the other hand, remained unchanged both with the [^{32}P]-labeled mutated 340-bp and with 340-bp wild type in the presence of molar excess of unlabeled AP-1 element (Fig. 6), suggesting that the binding of T_3 receptor type β to 340-bp is independent of AP-1 response element. The T_3-induced expression of nuclear proteins with binding affinity for AP-1 response element was further confirmed by additional gel shift analyses demonstrating the T_3-induced binding of nuclear proteins to a [^{32}P]-labeled double stranded oligonucleotide containing an AP-1 element. This binding was competed away by molar excess of unlabeled AP-1 oligonucleotide and a 146-bp PCR-generated promoter fragment (−28/+115) containing the AP-1 element (+92/+97) (Fig. 7). A PCR-generated 119-bp promoter fragment (−28/+91) lacking the AP-1 element was not able to compete with [^{32}P]-labeled double stranded oligonucleotide. Similar results obtained with the nuclear proteins of PMA (strong inducer of AP-1 expression)-treated cardiac fibroblasts (data not shown).

The role of AP-1 response element in T_3-induced inhibition of promoter activity

To determine the necessity of protein binding to the AP-1 response element on proximal sequences of pro α_1(I) collagen promoter for T_3-induced inhibition of promoter activity, we transiently transfected cardiac fibroblasts with CAT-linked constructs containing wild type −225/+115 sequences of pro α_1(I) collagen promoter and its mutated version in which specific base mutation (a C at position +93

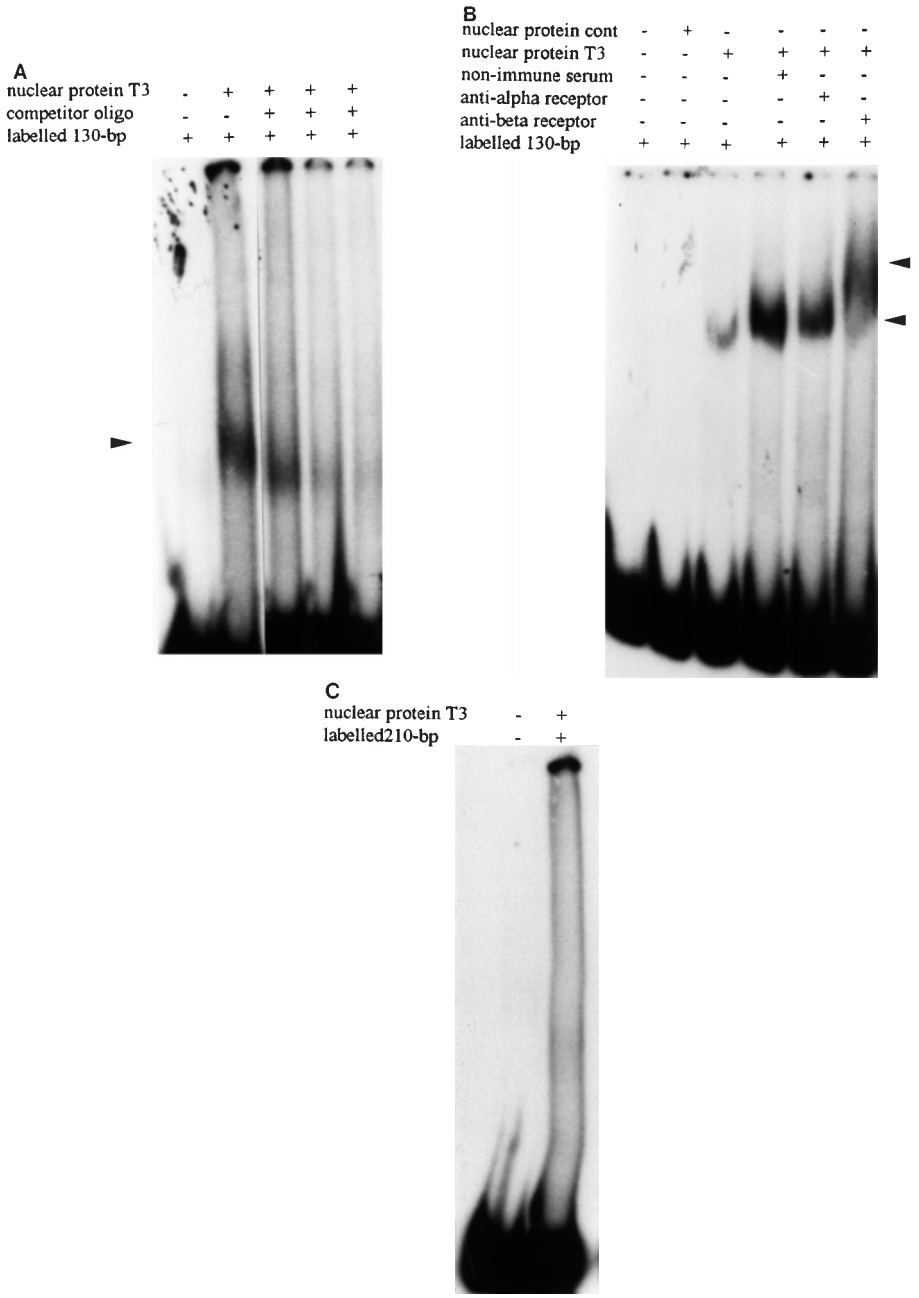

Figure 4 Gel mobility shift assay with non-overlapping fragments of *APa*I digest of the 340-bp (−225/+115) and nuclear proteins of cardiac fibroblasts. Nuclear proteins (6 μg) obtained from T_3-treated (10 nM, 24 h) were incubated with [γ-^{32}P]labeled (5 fmol) 130-bp (−15/+115) (panels A and B) and 210-bp (−225/−15) (panel C) subfragments of pro α_1(I) collagen promoter as described in Methods. Molar excess of an unlabeled double stranded oligonucleotide consisting of the thyroid hormone response element on malic enzyme gene was used in competition experiments. Anti-type α and β receptor polyclonal antibodies and non-immune serum were added (1 μg) to the binding reaction 1 h prior to the addition of radiolabeled DNAs. Electrophoresis was performed on a non-denaturing 3.5% polyacrylamide gel. These autoradiographs are representatives of three independent experiments.

was substituted by a G and a G at position +97 was substituted by a C) was introduced at the AP-1 site. The results of CAT assay showed that CAT expression in cardiac fibroblasts harboring the wild type construct was significantly reduced in T_3-treated cells (Fig. 8), whereas CAT activity in cardiac fibroblasts harboring the mutated constructs was not affected by T_3-treatment, suggesting that the AP-1 binding site located at +92/+97 and subsequently protein-binding to this site (as shown

LOCATION OF PUTATIVE CORE BINDING MOTIF OF TRE ON PRO α_1(I) COLLAGEN PROMOTER

Promoter Location	Sequence
-1406/-1385	-AGGACACTGTCTTTTAGGGAC-
-1290/-1267	-AGGACATGAGGAGTAGCCTTC-
-564/-542	-AGGTCTTCCCAAGGATGCCAG-

LOCATION OF AP-1 CONSENSUS SEQUENCE ON PRO α_1(I) COLLAGEN PROMOTER

Promoter Location	Sequence
-2254/-2246	-GGAGTCA-
-1143/-1139	-GGAGTCA-
-515/-511	-ACAGTCA-
+92/+97	-AGAGTCT-

Figure 5 Sequence analysis of pro α_1(I) collagen promoter for location of putative T_3-binding motif and AP-1 response elements.

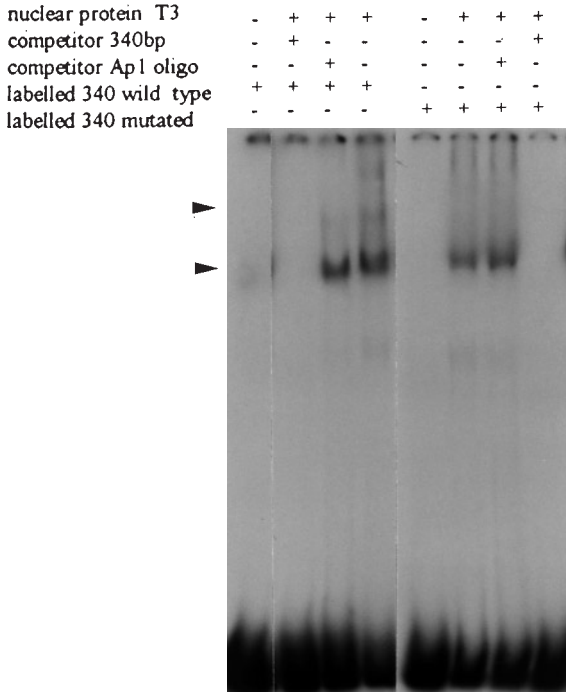

Figure 6 Gel mobility shift analysis with PCR-generated 340-bp (−225 to +115) wild type and its mutated version. Nuclear proteins (6 μg) from T_3-treated (10 nM, 24 h) cells were incubated with 5 fmol γ-[^{32}P]labeled PCR-generated wild type and mutated 340-bp (−225/+115) fragment of promoter and analyzed by gel mobility shift as described in Methods. Fifty-fold excess of unlabeled 340-bp wild type and a double-stranded oligonucleotide containing the AP-1 response element were used as unlabeled competitors. Electrophoresis was performed on a non-denaturing 4.5% polyacrylamide gel. These autoradiographs are representatives of three independent experiments.

by gel shift analysis) is necessary for T_3-induced inhibition of pro α_1(I) collagen promoter activity.

Discussion

In this study we have identified, for the first time, the sequences on pro α_1(I) collagen gene that are involved in the inhibition of collagen type 1 promoter in response to thyroid hormones. Specifically, we have demonstrated that thyroid hormone receptor type β binds to proximal (−15/+115) sequences of pro α_1(I) collagen promoter and that an AP-1 response element located at +92/+97 is necessary for the T_3-induced inhibition of pro α_1(I) collagen promoter activity in cardiac fibroblasts. Our results clearly demonstrate that while thyroid hormone-treatment of cells induces binding of receptor type β to proximal sequences of collagen type I promoter, this binding in the absence of AP-1 site (as seen with the mutated promoter sequences) is not sufficient for T_3-induced inhibition of promoter activity. These results are consistent with previously established concept that, in addition to their receptor-mediated interaction with the promoter of target genes, thyroid hormones exert their regulatory effects on gene transcription via pathways independent of the binding of thyroid hormone receptors to thyroid hormone response elements. In fact thyroid hormone receptors can cross talk with other transcription factors and regulate their activity and expression, thereby their respective signal transduction pathways. An example is their interaction with the components of AP-1 transcription factor, c-fos and c-jun protooncogenes (Zhang *et al.*, 1991). Using transient transfection it has been shown that the expression of c-jun and c-fos inhibits the thyroid hormone-dependent activation of thyroid hormone receptor types α and β and that thyroid hormone-receptors, in turn, inhibit the induction of c-fos promoter and regress the AP-1 site-dependent gene activation in HeLa and CV-1 cells (Zhang *et al.*, 1991). In separate studies it was shown that the functional interaction of c-erbA protein with c-jun enhances the transcriptional activity of both factors (Sharif and Privalsky, 1992). The interaction of components of AP-1 with thyroid hormone receptors in thyroid hormone-induced inhibition of thyrotropin β subunit gene has been established in a thyroid hormone receptor-deficient human placental cell line (Bodenner *et al.*, 1992) and in CV-1 cells (Wondisford

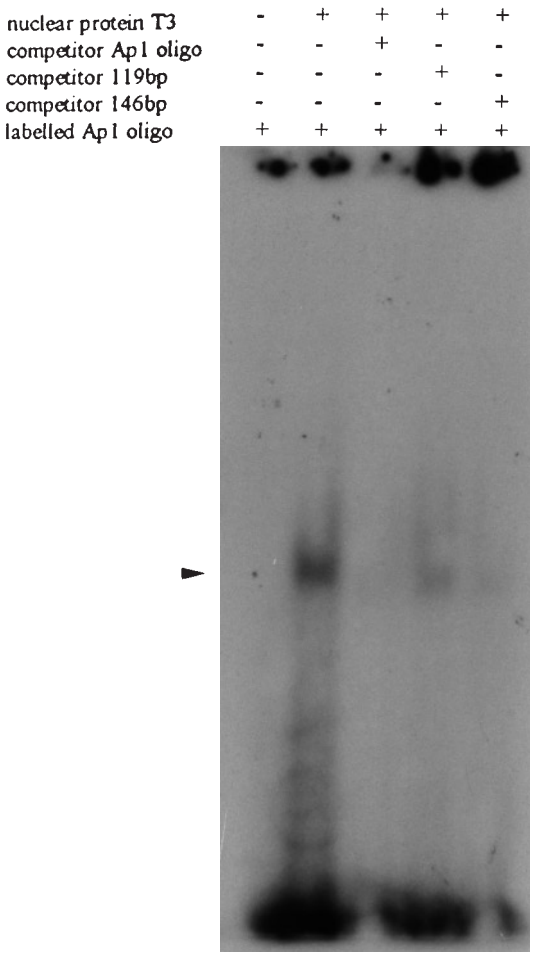

Figure 7 Thyroid hormone-induced binding of nuclear proteins to AP-1 response element. Nuclear proteins (6 μg) obtained from T_3-treated (10 nM, 3 h) cells were incubated with γ-[^{32}P]labeled (5 fmol) oligonucleotide containing the AP-1 consensus sequence as described in Methods. An unlabeled (50-fold) double stranded oligonucleotide consisting of the AP-1 response element and two PCR-generated promoter fragments, a 119-bp (−28/+91) lacking the AP-1 element and a 146-bp (−28/+115) containing the AP-1 elements were used in competition experiments. Electrophoresis was performed on a non-denaturing 3.5% polyacrylamide gel. These autoradiographs are representatives of three independent experiments.

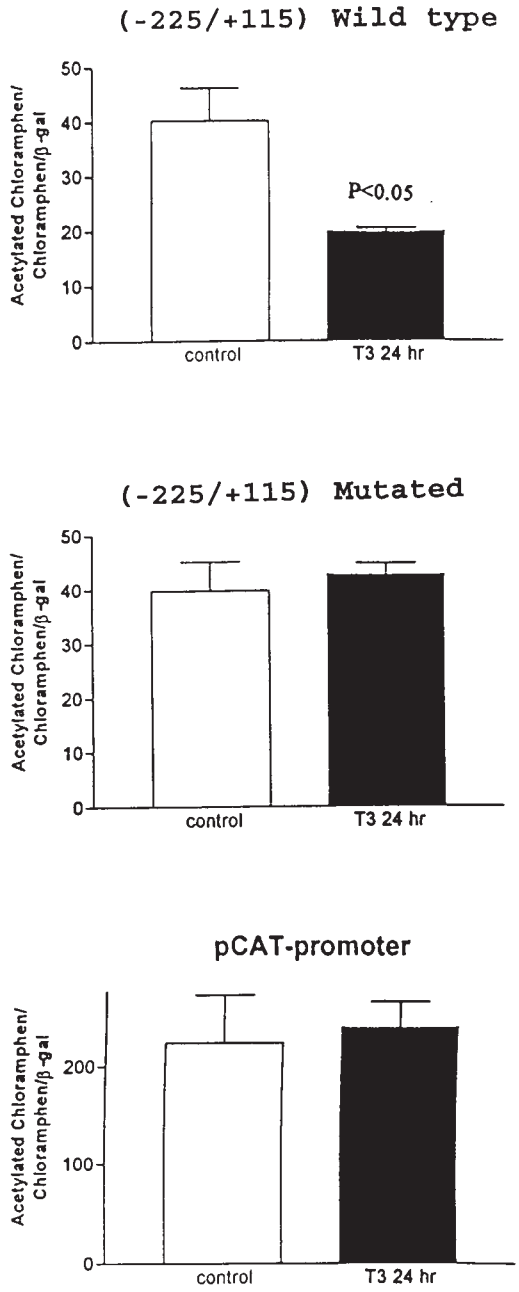

Figure 8 The effect of thyroid hormone on CAT expression under the control of wild type and mutated collagen type I promoter in cardiac fibroblasts. Cardiac fibroblasts were transiently transfected with plasmids containing CAT-linked deletion sequence (−225/+115) of pro α_1(I) collagen promoter and its mutated version in which the AP-1 response element (+92/+97) was mutated by specific base mutation (the C at position +93 was substituted by a G and the G at position +97 was substituted by a C). The plasmid pSV β-galactosidase was used as an internal standard for transfection efficiency. The effect of thyroid hormone (10 nM, 24 h) on expression of CAT in cytoplasmic extracts of fibroblasts harboring these constructs was determined as described in Methods. The effect of thyroid hormone on basic CAT expression was determined by using cytoplasmic extracts obtained from cardiac fibroblasts harboring a pCAT^{TM}-promoter plasmid. *t* statistics, $n=3$.

et al., 1993). Using dominant negative and non-dominant negative thyroid hormone receptors cloned from patients with thyroid hormone-resistance syndrome the interaction of thyroid hormone receptors with AP-1 in regulation of collagenase gene expression has been established (Kirk Ways *et al.*, 1993).

The role of AP-1 in regulation of collagen type 1 gene expression is well established. In liver Ito cells, an AP-1 site in the enhancer region of collagen type I gene (+598/+604) acts as the mediator in

transactivation of transforming growth factor-beta (TGF-β_1) (Armendariz-Borunda *et al.*, 1994). Katai *et al.* (1992) have shown that an AP-1-like motif in the intronic sequences of human pro α_1(I) collagen gene is involved in the transcriptional activity of the gene. In cardiac fibroblasts of the tight skin mouse, a genetic model of cardiac fibrosis, it was shown that a reduced interaction of negative regulatory sequences on pro α_1(I) collagen gene with AP-1 transcription factor is responsible for increased collagen type I gene expression (Phillips *et al.*, 1995). The results of the present study demonstrating the necessity of an AP-1 response element for thyroid hormone-induced inhibition of pro α_1(I) collagen promoter activity are consistent with an inhibitory function associated with the AP-1 response element located at $+92/+97$ on collagen type I promoter. Whether both components of AP-1, c-fos and c-jun, are involved in this inhibitory function must be determined. The results of this study also indicate that while the absence of protein binding to an AP-1 element, as achieved by introduction of mutation in the AP-1 binding site, does not affect the binding of thyroid hormone receptor type β to the promoter sequences, it eliminates the T_3-induced inhibition of promoter activity. It is, therefore, important to investigate the nature of an interaction between the AP-1 components and the thyroid hormone receptor type β in regulation of collagen type 1 gene expression in cardiac fibroblasts.

Combined presence of positive and negative thyroid hormone response elements on the promoter of many genes including rat growth hormone (Crone *et al.*, 1990) and myosin heavy chain genes (Izumo *et al.*, 1986) have been demonstrated. It is also established that depending on the tissue in which they are expressed, these genes may be regulated by thyroid hormones in opposite directions (Izumo *et al.*, 1986). The core binding motif for positive T_3 response element is *AGGTCA* N_x *AGGTCA* or the variations of the inverted repeat (Umesono *et al.*, 1991). Although a universal core binding motif for negative thyroid hormone response elements is not identified, they are usually located in the regions adjacent to the transcription initiation site on the target genes. A position-independent negative T_3 response element on thyrotropin promoter adjacent to the transcription initiation site ($+11/+27$) has been also shown to be involved in the negative regulation of thyrotropin gene by thyroid hormones (Carr *et al.*, 1992). In this study, sequence analysis of the 3.6 kb upstream segment of rat pro α_1(I) collagen promoter for positive T_3 response elements led to the identification of three fragments ($-564/542$, $-1290/-1267$, and $-1406/-1385$) containing the two half sites in the core binding motif of positive T_3 response element (Fig. 4). Although our data demonstrate the binding of thyroid hormone receptor to $-15/+115$ segments of the promoter, the positive core binding sites were not identified in those regions. This is consistent with the fact that unlike response elements consensus sequences for negative T_3 response elements have not been identified. The results of sequence analysis also revealed three Ap-1 binding sites on the upstream regions of the promoter. These findings, together with other results of present study, point to the existence of multiple sites on the pro α_1(I) collagen promoter to which thyroid hormone receptors and thyroid hormone-induced transcription factors, including Ap-1, can bind. For a complete understanding of the inhibition of collagen gene expression by thyroid hormone, future studies must identify the function of T_3 and AP-1 response elements and determine if they act in concert or in opposite directions. Do they act via binding to one or multiple thyroid hormone-induced proteins?

The environment of the cells in a specific tissue can alter the biological response to hormones and growth factors. Collagen type I gene expression may be differentially regulated by diverse intrinsic micro-environments in various tissues. This phenomenon has been well established in osteoblastic fibroblasts in which the stimulatory elements located between -3521 and -2295 on the 3.6 kb pro α_1(I) collagen promoter are preferentially active (Pavlin et al., 1992). Future studies must therefore determine if thyroid-hormone-induced inhibition of pro α_1(I) collagen is cardiac-specific and if not, are there differences between pathways controlling the T_3-induced regulation of collagen gene expression in cardiac and non-cardiac cells?

In summary, these results identify parts of the pathways by which thyroid hormones exert their inhibitory effect on collagen type I gene expression. In healthy individuals the inhibitory effects of thyroid hormones may contribute to the normal regulatory mechanisms controlling collagen matrix biosynthesis. However, in cases of thyroid gland malfunction, these inhibitory effects, if not compensated or neutralized, may lead to disproportionate collegen production and abnormalities of extracellular matrix which, in turn, contribute to cardiac manifestations. The mechanisms of thyroid hormone-induced inhibition of collagen type I gene expression may be clinically exploited to design preventive and therapeutic strategies to overcome excessive collagen synthesis which is

associated with almost all types of ventricular hypertrophy.

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