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Molecular characterization of myocardial fibrosis during hypothyroidism: evidence for negative regulation of the pro-α1(I) collagen gene expression by thyroid hormone receptor

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Abstract

The purpose of this study was to gain insights into the underlying mechanism of myocardial fibrosis during hypothyroidism. Treatment of cardiac fibroblasts with a medium lacking thyroid hormone led to a 47% increase in [^{3}H]thymidine incorporation into the cell nuclei compared with that in untreated cells. Northern blot analysis of RNA from cardiac fibroblasts grown in a thyroid hormone depleted medium resulted in a 38% increase in the abundance of mRNA for pro-α1(I) collagen. At the protein level, the amount of type I collagen, as determined by immunoprecipitation, was increased either in the cell lysate (46%) of cardiac fibroblasts grown in a thyroid hormone depleted medium or in the medium (44%). The chimeric plasmid, ColCAT 3.6, contains the 5′-flanking region of the rat pro-αl(I) collagen gene (from bases -3520 to $+115$) fused to the chloramphenicol acetyltransferase (CAT) gene. The plasmid was cotransfected with thyroid hormone receptor (TR) expression plasmid into rat cardiac fibroblasts and COS-l cells (monkey mesangial cells). Cells transfected with the ColCAT plasmid in the presence of thyroid hormone (100 nM T_3) had a significant decrease (39% in fibroblasts, $P < 0.01$; 52% in COS-1 cells, $P < 0.001$) in CAT activity when compared to cells not exposed to thyroid hormone. Transient co-transfection of TR with various pro-αl(I) collagen/CAT deletion constructs showed that T_3-dependent repression was preserved with the deletion from 3520 bp of the flanking sequence to a 5′ end point at position -224, indicating that a thyroid hormone-response element (TRE) was localized at the region -224 to $+115$. The TR-DNA binding assays demonstrated binding of the human TRβ1 to a fragment containing a proposed TRE located between position -35 and $+115$ in the 5′-flanking region of the rat pro-αl(I) collagen gene.

Keywords: Thyroid hormone; Thyroid hormone receptor; Hypothyroidism; Myocardial fibrosis; Thyroid hormone-response element; Pro-α1(I) collagen gene

1. Introduction

It has been shown that once myocyte proliferation ceases shortly after birth in the mammalian heart, both physiological and induced myocardial growth occur primarily through hypertrophy of myocytes (Anversa et al., 1986). In contrast, a hyperplastic component persists in interstitial fibroblasts and capillary endothelial cells which, in combination with cellular hypertrophy, participates in the expansion of the non-myocyte compartment in the overloaded myocardium. The reactive interstitial and perivascular fibrosis accounts for abnormal myocardial stiffness and ultimately ventricular diastolic dysfunction and is likely a result of cardiac fibroblasts growth and enhanced collagen synthesis (Weber and Brilla, 1991). Thus, detailed characterization of the mechanism involved in myocardial fibrosis is of great importance.

Regulatory effects of thyroid hormone (T_3; 3,5,3′-L-triiodothyronine) on cardiac myocytes and on contractile protein gene expression in the heart have been well established (Izumo and Mahdavi, 1988; Tsika et al., 1990). However, relatively little is known about the effect of thyroid hormone on cardiac interstitium and

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interstitial protein genes expression. Decreased collagen gene expression and absence of fibrosis in thyroid hormone induced myocardial hypertrophy have been reported (Yao and Eghbali, 1992). A recent study demonstrated that the synthesis and deposition of type I collagen increase in thyroidectomized rat hearts (Klein et al., 1996). In hypothyroidism and myxedema, diastolic abnormalities of the heart have been found either from an echocardiographic study (Santos et al., 1980) or from a clinical observation (Mac Kerrow et al., 1992). Together, these findings suggest that thyroid hormone plays a role in the development of myocardial fibrosis in vivo. However, in vivo model of hypothyroidium is activated in situations associated with hemodynamic changes. This may arise, in part, from activation of reflex neuroendocrine activity which accompanies hemodynamic changes (Polikar et al., 1993). Cardiac fibroblasts, which are the cellular origin of collagen matrix in the heart, constitute the major component of non-myocyte cells (Eghbali et al., 1988). Therefore, it is important to determine whether a thyroid hormone depleted condition affects the growth of cardiac fibroblasts in culture.

Thyroid hormone regulates development and metabolism, acting via a family of thyroid hormone receptors (TR) to modulate the transcription of thyroid hormone-responsive genes. In current models for thyroid hormone action, T_3 binds to its intranuclear receptors to either stimulate or repress transcriptional activity by interactions with specific thyroid hormone-response elements (TREs) of target genes (Evans, 1988; Samuels et al., 1988). Thyroid hormone receptors are members of a superfamily of receptors that also include the steroid hormones, vitamin D and 9-*cis*-retinoic acid receptors (RXRs). The TR requires RXR, as an auxiliary protein, to bind to their TREs efficiently (Evans, 1988). Two major subtypes of TR, designated α (chromosome 17) (Sap et al., 1986) and, β (chromosome 3) (Weinberger et al., 1986), have been described.

Type I collagen is the most abundant fibrillar collagen found in the heart (Eghbali and Weber, 1990). The synthesis of type I collagen can be modified by a variety of hormones and pathological situations by mechanisms acting mainly at the transcriptional level (Vuorio and de Crombrugghe, 1990; Slack et al., 1993). To understand the complex hormonal regulation and its alterations, studies aiming at identifying *cis*-acting TRE in the pro-αl(I) collagen gene are of considerable interest. Therefore, in the present study, we have examined the changes of gene expression by thyroid hormone in cultured cardiac fibroblasts implicated in myocardial fibrosis. We have focused specifically on the transcriptional regulation of the pro-α1(I) collagen gene by thyroid hormone using the transient transfection system. In addition, we have measured the transcriptional activity of the 5′-flanking pro-αl(I) collagen genomic deletion constructs cotransfected with TR expression plasmids. Transfection and DNA binding studies revealed that the pro-α1(I) collagen gene contains a binding site for the receptor which functions as a TRE.

2. Materials and methods

2.1. Cell culture

Cultured cardiac fibroblasts were yielded as described previously (Eghbali et al., 1991; Bashey et al., 1992). Adult (Wistar) rats, weighing about 200 g, were anesthetized; hearts were excised, minced and washed in Dulbecco's modified Eagle's medium (DMEM). The tissue was then subjected to digestion at 37°C by a mixture of 0.1% trypsin in Hank's balanced salt solution (HBSS) and 100 units/ml collagenase (type IV, Sigma) for 10 min. Cells from the second to ninth digestion were plated on 100 mm culture dishes in DMEM plus 10% fetal bovine serum (FBS) and incubated for 3 h at 37°C in a humidified incubator with 5% CO_2. At the end of this period, attached cells (mostly fibroblasts) were grown in DMEM plus 10% FBS. The fibroblastic nature of cells was determined by immunofluorescence staining with anti-human factor VIII for endothelial cell, anti-desmin for muscle cells and anti-vimentin for fibroblasts. In early passages, only 1–2% of cells stained positively with anti-factor VIII and anti-desmin. In all of our studies, only cells from passages 2–4 were used. Cos-1 cell lines (monkey mesangial cells) were maintained in DMEM plus 10% FBS.

2.2. Incorporation of [^{3}H]thymidine

Cardiac fibroblasts were grown in DMEM containing 10% FBS until confluent on 35 mm dishes. Cells were then deprived of serum for 36 h. Fresh medium containing 20% FBS, thyroid hormone depleted (Td) serum and Td plus various concentrations of T_3 serum were added. The Td serum was prepared as described (Samuels et al., 1979). For determining the change of cell cycle, cells were labeled with [^{3}H]thymidine at a concentration of 25 μCi/ml for 30 min at selected time points after synchronization. After labeling, cells were rinsed with phosphate-buffered saline (PBS) twice and lysed with 1 ml of 0.1 M NaOH containing 10 mM EDTA and 0.5% SDS. After being heated at 70°C for 30 min, the cell lysate was mixed with 100μl of 100% trichloroacctic acid (TCA) and incubated at 4°C overnight. The TCA precipitates were then collected on GF/C filter discs (Millipore), rinsed with 5% TCA solution and then with 95% ethanol, air-dried and scintillation-counted. For determining total amount of DNA synthesis, 5 μCi/ml of [^{3}H]thymidine was added from 12 to 24 h after synchronization. DNA concentra-

tion in the cell lysate was measured by the use of chromogenic reagent (0.7 M 3,5-diaminobenzoic acid) and counted by spectrophotometry using 410 and 500 nm as the exciting and emission wavelength, respectively (Manford and Patterson, 1979).

2.3. RNA extraction and Northern blot analyses

Total cellular RNA was isolated from cardiac fibroblasts. Cultures were initiated by seeding 5×10^6 cells/100 mm culture dish and were grown under the culture medium containing 20% FBS, thyroid hormone depleted (Td) serum and Td plus various concentrations of T_3 serum for 48 h. In each experiment, cells were pooled from two to three dishes/culture condition. Total cellular RNA was extracted using guanidinium isothiocyanate essentially as described (Chomczynski and Sacchi, 1987). For Northern blot analysis, 10 μg of total RNA was size fractionated in 1% agarose gels containing 1 M formaldehyde. The agarose gel was blotted onto nylon membrane (Schleicher and Schuell) using an electro-transfer apparatus (Hoefer Scientific Instruments TE62). A recombinant plasmid containing rat pro-α1(I) collagen sequences that covered the entire 3′ non-coding and about half of the C-terminal propeptide region of pro-α1(I) collagen mRNA (kind gift of Genovese et al., 1984) was used as a probe. A full length cDNA for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The filters were hybridized with DNA fragments labeled with [^{32}P]-labeled random primed extended products and were exposed to X-AR5 (Kodak) film. Quantitation of the hybridizing bands from the autoradiograms was analyzed using MasBas software (Fujifilm, Japan). The density of an individual mRNA band was divided by the density of the corresponding GAPDH band on the membrane.

2.4. Immunoprecipitation

Metabolic labeling of cardiac fibroblasts with [^{35}S]methionine was performed as described previously (Lin et al., 1991; Bashey et al., 1992). Briefly, fibroblasts were plated in DMEM containing 10% FBS in l00 mm dishes. When cells were subconfluent, the medium was aspirated and cells were incubated with DMEM containing 20% FBS, Td serum and Td plus 10^{-6} M T_3 serum for 24 h. Labelling with [^{35}S]methionine was performed in 3 ml of methionine-free medium containing the above sera and 100 μCi/ml of [^{35}S]methionine. After incubation for 5 h at 37°C, cells were rinsed with ice cold PBS and lysed with 0.5 ml of EBC buffer (50 mM Tris–HCl/pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40) at 4°C for 10 min. Radioactivity incorporated into each sample was determined by measuring the TCA precipitable counts. Aliquots of the cell lysates and culture media containing equal radioactivity were immunoprecipitated with antibodies against TRβ1 (J52, a monoclonal antibody) (Lin et al., 1990), TR α (Affinity Bioreagents) and rat type I collagen (Chemicon). Preimmune rabbit serum was incubated as control. Fifty microliters of washed *Staphylococcus aureus* in suspension was added and incubated at RT for 30 min. Immunoprecipitates were collected by centrifugation and washed 6 times with a solution containing 0.16 M NaCl, 0.5% Nonidet P-40 and 0.1% SDS. Laemmli gel sample buffer (30 μ1) was added to the immunoprecipitates, boiled at 100°C for 5 min, analyzed on SDS-PAGE and fluorographed.

2.5. Plasmid construction

The plasmid ColCAT 3.6 contains 3520 bp of rat pro-αl(I) collagen promoter followed by 115 bp of the rat αl(I) first exon cloned upstream of chloramphenicol acetyltransferase (CAT) gene within the pUC 12 vector, as described elsewhere (generous gift of Dr David Rowe, University of Connecticut) (Lichtler et al., 1989). The plasmids pCLC51 and pCLC61 were derived from human TRβ1 and TRαl expression vectors, as described previously (Lin et al., 1992). The plasmid pTreTK28CAT contains a copy of palindromic elements of positive TRE inserted upstream of the thymidine kinase (TK) promoter and CAT gene (generous gift of Dr David Moore, Massachusetts General Hospital, Boston) (Brent et al., 1989). A promoterless CAT plasmid, p0CAT (which was prepared by removing the collagen promoter fragment with *Xba*I from ColCAT 3.6 and religated) was used as a negative control. The plasmid pTKCAT (which was prepared by replacing the 3635 bpα1 collagen promoter fragment with TK promoter in ColCAT 3.6) was used as a positive control.

Several smaller constructs were made by deletion of fragments from ColCAT 3.6 plasmid, as described previously (Ritzenthaler et al., 1991). Briefly, as indicated in Fig. 6, ColCAT 2.4, ColCAT 1.7, ColCAT 1.0, ColCAT 0.4 and ColCAT 0.3 were yielded by a series digestion of the 5′-flanking region of the ratα1(I) collagen gene using *Hind*III, *Pst*I, *Stu*I, *PpuM*I and *Bgl*II, respectively.

2.6. Transient DNA transfection and enzymatic assays

Transient transfection was carried out by the lipofectamine (Life Technologies) according to the manufacturer's instructions. Briefly, a 2×10^5 cells/35 mm dish were plated in 10% FBS serum and incubated until 80% confluent. For each transfection, cells were transfected with 1 μg of reporter plasmid, 1 μg TR and 0.5 μg pCMV-β-gal in serum-free Opi-MEM (Life Technologies). After incubation with 16 μg of lipofectamine for

5 h at 37°C in a CO_2 incubator, cells were washed and changed to the appropriate serum-containing medium. After an additional 48 h, the cell lysates were harvested with buffer containing 100 mM Tris–HCl, pH 7.8, 0.5% Triton X-100 and fractured by freeze-thawing. Extracts were normalized for protein contents as measured by Bradford's method (Bradford, 1976) and incubated with (^{14}C) acetyl-CoA and chloramphenicol overnight at 37°C. Acetyl chloramphenicol was extracted using organic solvent and quantitated by the β-scintillation counting (Gorman et al., 1982). The CAT activity was normalized with the β -galactosidase activity.

2.7. Electrophoretic mobility shift assays

Two probes for eletrophoretic mobility shift assays were prepared by enzymatic digestion of the ColCAT 0.3 plasmid with *Bgl*II, *Bstx*I and *Xba*I, respectively. The resulting 189 bp *Bgl*II–*Bstx*I and 150 bp *Bstx*I–*Xba*I fragments were purified and end-labeled with [(α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. Competition studies were performed with 20-fold molar excesses of unlabeled DNA fragments or non-specific oligonucleotides. Human TRβ1 protein was expressed in *Escherichia coli* BL 21 and purified as described (Lin et al., 1990). The RXR β cDNA (pTL1/HRXRβ, Pierre Chambon, Universite Louis Pasteur, France) was in vitro transcribed and translated using the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol. Purified h-TRβ1 (1 μg) and/or RXR were incubated with 50 000 cpm of labeled probe in 20 μ1 buffer containing 25 mM HEPES (pH7.5), 5 mM $MgCl_2$, 4 mM EDTA, 2 mM dithiothreitol, 110 mM NaCl, 5 μg/ml bovine serum albumin, 3 mg/ml poly(dI-dC) (Pharmacia LKB Biotechnology) and 0.1 μg of salmon sperm DNA (Sigma) for 30 min at RT. After incubation, samples were then subjected to electrophoresis on 5% polyacrylamide gels in 0.5 X TBE buffer (45 mM Tris borate and 1 mM EDTA) for 3–4 h at 4°C. The gel was dried and autoradiographed with an intensified screen.

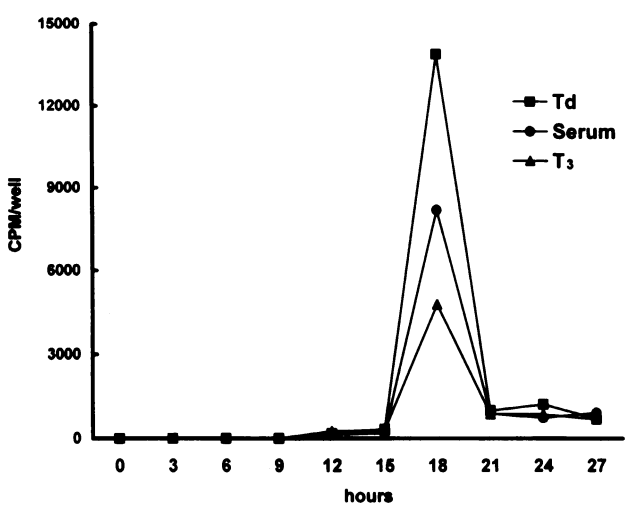

Fig. 1. Kinetics of [^{3}H]thymidine incorporation into DNA in rat cardiac fibroblasts cultured in the media containing regular serum (Serum), thyroid hormone depleted serum (Td) and Td plus 10^{-6} M T_3 serum. Prior to the experiments, the cultures were incubated for 36 h in serum-free medium. At time 0, the following additions were made: regular serum (20% FBS), 20% Td serum and Td plus 10^{-6} M T_3 serum. At the indicated times after the addition of the above sera [^{3}H]thymidine (25 μCi) was added to each well and after 30 min at 37°C, the cell lysates with TCA precipitated were counted as described in Section 2. The data are representative of three independent experiments.

2.8. Statistical analysis

[^{3}H]thymidine incorporation and CAT activity were expressed as a mean ± standard deviation (S.D.). Differences between two groups were determined using unpaired *t*-test. For multiple groups, one-way analysis of variance (ANOVA) was performed and followed by either post hoc Dunnett or Scheffe multiple-comparison procedures. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effect of thyroid hormone on cellular proliferation

The effect of thyroid hormone on proliferative capacity of cardiac fibroblasts was determined by the extent of [^{3}H]thymidine incorporation into DNA. We examined the kinetics of [^{3}H]thymidine incorporation into DNA in rat cardiac fibroblasts treated with various concentrations of thyroid hormone. [^{3}H]thymidine incorporation reached a peak at 18 h. Furthermore, there were no changes in the progression of the cell cycle under different concentrations of thyroid hormone (Fig. 1). A thyroid hormone depleted condition enhanced DNA synthesis significantly (47%, $P < 0.05$) in cardiac fibroblasts compared with that in cells grown under regular serum and Td plus physiological concentrations (10^{-9}–10^{-7} M) of T_3 serum. The activation of [^{3}H]thymidine incorporation was inversely dependent on the concentration of T_3; maximal effect at 10^{-7}–10^{-6} M (Fig. 2). The results suggest that a thyroid hormone depleted condition promotes proliferation of rat cardiac fibroblasts.

3.2. Effect of thyroid hormone on the biosynthesis of type I collagen

The expression of extracellular matrix protein genes was determined by Northern blot analysis at the RNA level and immunoprecipitation at the protein level. The

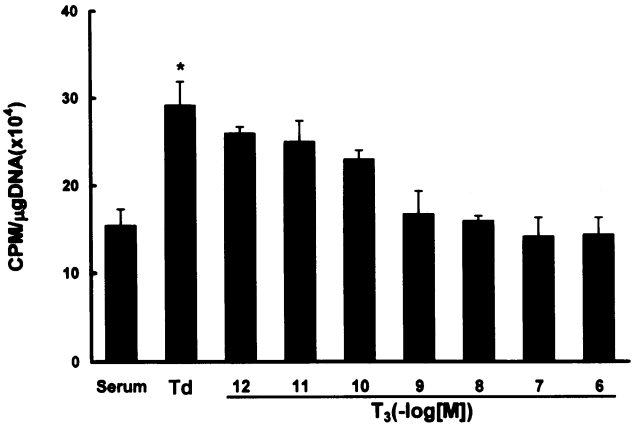

Fig. 2. Proliferative response of rat cardiac fibroblasts cultured in the media containing regular serum (Serum), thyroid hormone depleted serum (Td) and Td plus indicated concentrations of T_3 serum. Prior to the experiments, the cultures were incubated for 36 h in serum-free medium. The above sera were added to cardiac fibroblasts as described in Fig. 1. Cells were exposed to [^{3}H]thymidine (5 μCi) at 12–24 h after the addition of the above sera, incorporation of [^{3}H]thymidine into TCA precipitable material was determined as described in Section 2. All values are mean ± S.D., * indicates a difference from regular serum and Td plus 10^{-9}–10^{-6} M T_3 serum at 0.05 significant level.

results of Northern analysis for pro-α1(I) collagen mRNA level are shown in Fig. 3. Treatment of rat cardiac fibroblasts with the Td serum resulted in a 38% ($P < 0.05$) increase in the abundance of mRNA for pro-α1 (I) collagen. The addition of physiological concentrations (10^{-9}–10^{-7} M) of T_3 to the Td serum reversed this effect. The expression of pro-α1(I) collagen mRNA was inversely dependent on the concentration of T_3 ($r = -0.94$). There was no apparent effect of thyroid hormone on GAPDH gene transcription. To understand if hypothyroid-induced effects on type I collagen mRNA led to a parallel regulation of the level of type I collagen protein in cardiac fibroblasts, we determined the amount of immunoreactive type I collagen in the cell lysates and culture media by an anti-type I collagen antibody. Immunoprecipitation of radiolabeled collagens synthesized by cardiac fibroblasts and secreted into the culture media has been well described (Bashey et al., 1992). The level of immunoreactive rat type I collagen either in the cell lysates (46%, $P < 0.05$) or in the culture media (44%, $P < 0.05$) increased during a thyroid hormone depleted condition (Fig. 4a and b). The results were consistent with those obtained for Northern blot analysis.

3.3. Effect of thyroid hormone on the expression of thyroid hormone receptor gene in rat cardiac fibroblasts

As described previously, the level of expression of TRβ1 mRNA is unaffected by T_3 in the heart of intact rats (Lazar and Chin, 1990). Therefore, we wanted to determine whether rat cardiac fibroblasts express TR and to examine the effect of thyroid hormone on the expression of TR. Immunoprecipitation of the cell lysate with J52 antibody (anti-human TRβ1) confirmed the presence of TRβ1 in rat cardiac fibroblasts. The specificity of this antibody has been shown in our previous study (Lin et al., 1991). We also found that thyroid hormone, independent of thyroid status, had no effect on the expression of the TRβ1 gene (Fig. 4c). Moreover, thyroid hormone receptor α was undetectable by immunoprecipitation (data not shown). Thus, TRβ was expressed in rat cardiac fibroblasts and unaffected by thyroid hormone.

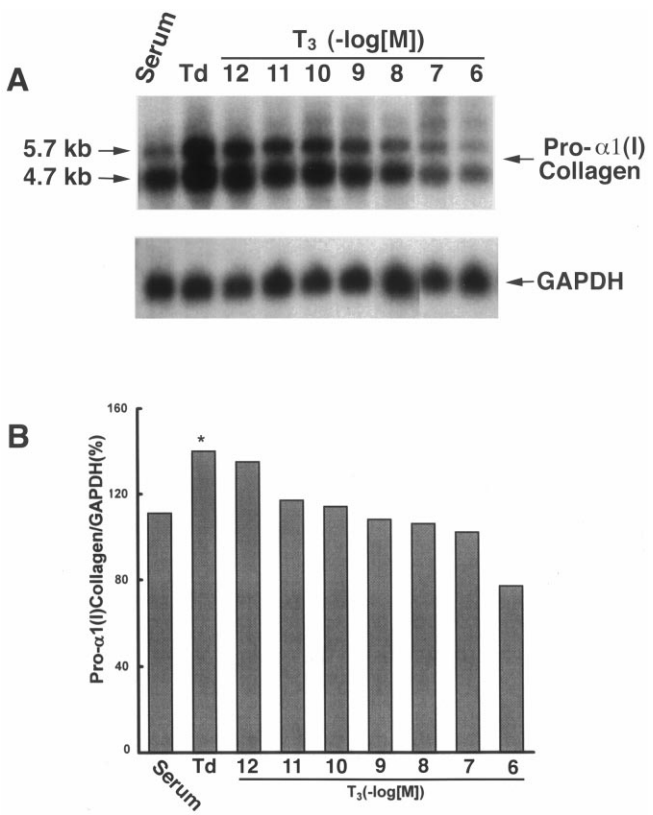

Fig. 3. Autoradiograms of Northern hybridization analysis of RNA from rat cardiac fibroblasts in culture. Total RNA (10 μg) from cardiac fibroblasts grown in the media containing regular serum (Serum), thyroid hormone depleted serum (Td) and Td plus indicated concentrations of T_3 was electrophoresed on a 1% agarose gel as described in Section 2. Panel A shows the mRNA for pro-α1(I) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In panel B, the levels of pro-α1(I) collagen mRNA were measured as radioactivities using an imaging analyzer, normalized relative to the radioactivity generated by probing for GAPDH and expressed relative to those achieved with RNA from cardiac fibroblasts grown in the media containing regular serum (100%). Bars represent means of three independent experiments. * indicates a difference from regular serum and Td plus 10^{-9}–10^{-6} M T_3 serum at 0.05 significant level.

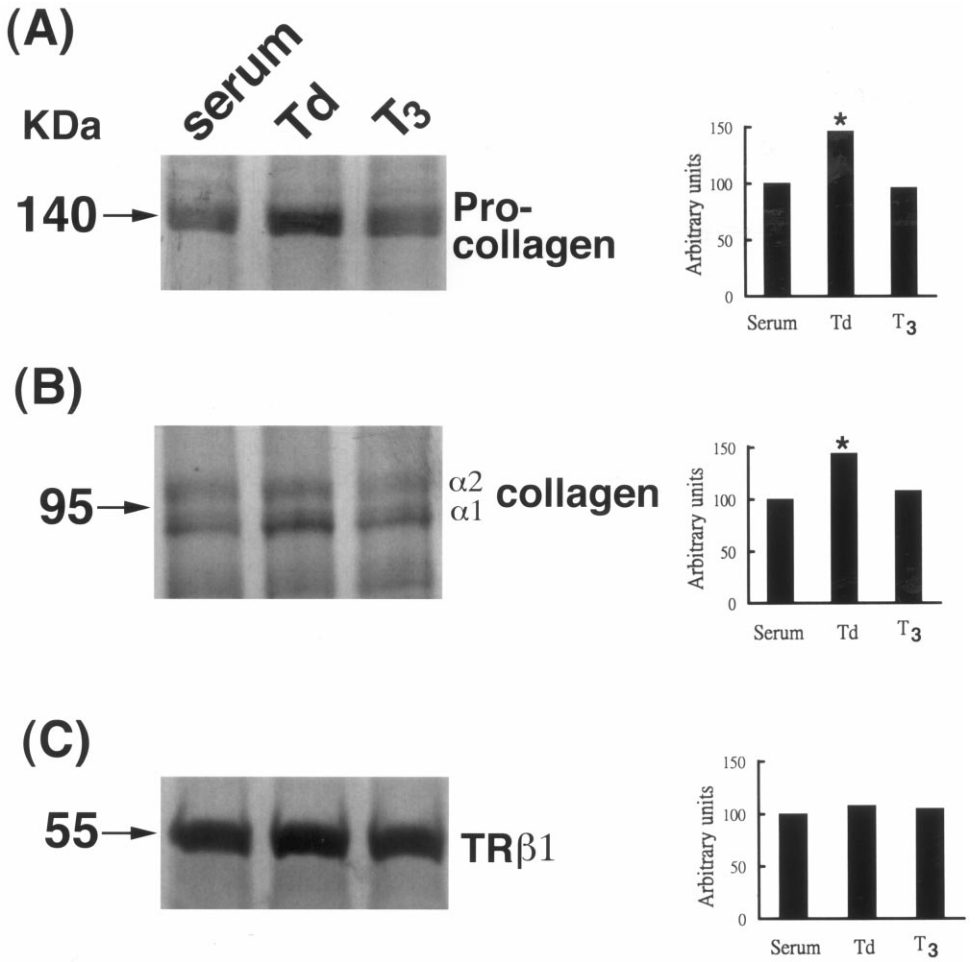

Fig. 4. Immunoprecipitation of immunoreactive proteins from the culture media and cell lysates of cultured cardiac fibroblasts. Rat cardiac fibroblasts were cultured in the media containing regular serum (Serum), thyroid hormone depleted serum (Td) and Td plus 10^{-6} M T_3 serum (T_3). Cells were metabolically labeled with [^{35}S]methionine. The media and cell lysates of cultured cardiac fibroblasts were immunoprecipitated with anti-type I collagen and thyroid hormone receptor (TR)β1 antibodies as described in Section 2. The immunoprecipitates were analyzed on a 10% SDS-PAGE. SDS-PAGE fluorographs of the immunoreactive type I collagen from the cell lysates (panel A) and media (panel B) are shown. Panel C shows specific immunoprecipitates of the cell lysates with TRβ1 antibody. The position of 140 KDa procollagen, 95 KDa collagen and 55 KDa TRβ1 bands are indicated. Bars represent means of three independent experiments. * indicates a difference from regular serum and Td plus 10^{-6} M T_3 serum at 0.05 significant level.

3.4. Thyroid hormone represses the pro-α1(I) collagen gene transcription

The next series of experiments was designed to determine if the repression of rat pro-αl(I) collagen mRNA levels by thyroid hormone was caused by decreased transcriptional activity of this gene. The recombinant plasmid, ColCAT 3.6, which contains the 3520 bp 5′-upstream flanking sequence of the rat pro-αl(I) collagen gene and 115 bp downstream from the transcription initiation site ($-3520-+115$ bp) linked to the CAT gene was transfected into two kinds of mammalial cells (rat cardiac fibroblasts and monkey COS-1 cells). COS- 1 cells have been shown to be deficient in endogenous functional TR expression (Lin et al., 1991) and are, therefore, amenable to co-transfection with TR expresssion plasmids. The resulting CAT expression was measured in the presence or absence of T_3. As shown in Fig. 5a and b, cotransfection of the TR expression plasmid in the presence of T_3 leads to repression of CAT activity either in rat cardiac fibroblasts (39%, $P < 0.01$) or in COS-1 cells (52%, $P < 0.001$). In addition, both TRβ and TRα lead to a similar repression of CAT activity. Whereas, in the absence of TR, no hormonal repression was observed.

3.5. Localization of the TRE in the rat pro-α1(I) collagen gene by deletion analysis

Since the transcriptional effects of thyroid hormone are mediated by the nuclear TR, we sought to determine whether the rat pro-α1(I) collagen gene contains TRE in its 5′-flanking region. For this purpose, six deletion mutants of the 5′-flanking region were used.

Each of these constructs shares a common 3′ end which lies 115 bp downstream of the transcription initiation site. They differ only in their 5′ extent. These fragments were fused to the CAT gene in the pUC12 expression vector. The smallest construct (ColCAT 0.3) which contains the CCAAT and TATA areas has been considered the proximal promoter for the rat pro-α1(I) collagen gene (Rippe et al., 1989). Cotransfection of the TRβ expression plasmid (pCLC51) and the various chimeric pro-α1(I) collagen /CAT deletion mutants were transiently expressed in COS-1 cells. Fig. 6 shows the relative CAT activities of the different deletion mutants and their level of trans-activation by TR in COS-I cells. T_3-dependent repression was preserved with the deletion from −3620 bp of the flanking sequence to −224. No thyroid hormone effect was noted in the positive control, pTKCAT, which contains the TK promoter, or the negative control, p0CAT, both of which lack the collagen promoter. The trans-activation studies indicate that the rat α1(I) collagen gene contains a TRE in the 5′-flanking region between −224 and +115 bp.

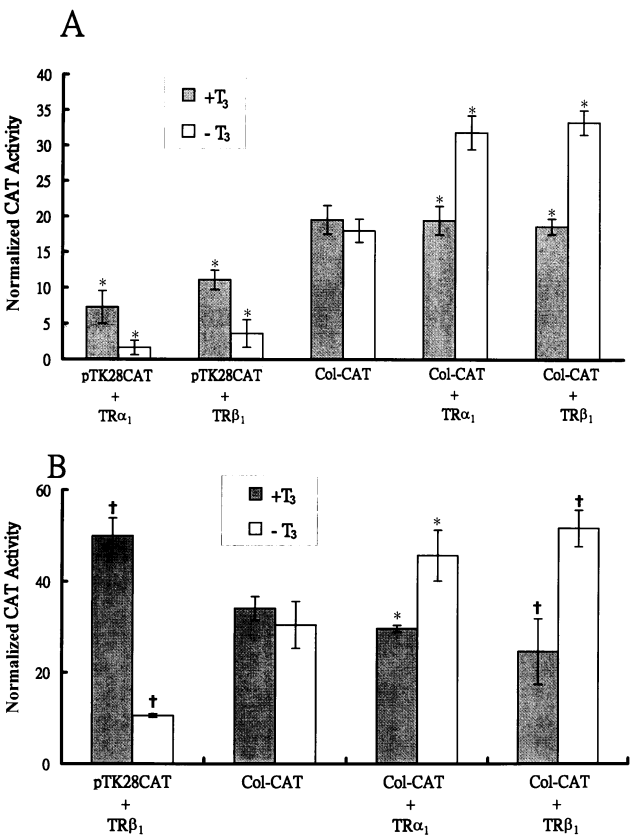

Fig. 5. Effect of T_3 on CAT activity driven by the 5′-flanking region of the pro-α1(I) collagen gene in cardiac fibroblasts and COS-1 cells. Panel A, Cardiac fibroblasts were co-transfected with ColCAT 3.6 containing the full length pro-α1(I) collagen promoter (−3620–+115) and the TR expression plasmid containing TRβ1 or TRα1 (pCLC51, 61) either in the presence (solid bars) or absence (open bars) of 100 nM T_3 by the lipofectamine method. The plasmid pTreTK28CAT was used as a positive control for CAT activity and trans-activation by TR. CAT activity in the cell extracts was assayed as decribed in Section 2. Values (mean ± S.D. of at least three independent experiments, with triplicate assays) are expressed as CAT-specific activity normalized by internal β-galactosidase activity (CAT activity/mg of protein/β-galactosidase activity) to correct for transfection efficiency. Panel B, An identical paradigm was followed using COS-1 cells. * $P < 0.01$, †$P < 0.001$ (t-test).

3.6. Delineation of T_3 receptor binding sites in the pro-α1(I) collagen gene

We next sought to determine whether the 5′-flanking region of the pro-α1 (I) collagen gene with TRE activity in the cotransfection experiments could bind to TR in vitro. We performed the gel shift TR-DNA binding assay to determine whether the in vitro translated TR protein showed specific binding to the 5′-flanking region of the pro-α1 (I) collagen gene. Our previous study showed that this in vitro translation system produces specific high affinity TR (Lin et al., 1990). Fig. 7 shows the results of an experiment in which the human TRβ1 synthesized in vitro was bound to DNA from the 5′-flanking region of the pro-α1(I) collagen gene. The following adjoining DNA fragments from the 5′ flanking region of the pro-α1(I) collagen gene were used as probes: (a) −224–−35 bp and (b) −35 and +115 bp. Those results demonstrated that the region between −35 and +115 bp contained a single well defined site capable of binding TRβ1 (Fig. 7, lane 2), while there was no binding of TRβ1 in the −224–−35 bp region (data not shown). As a negative control, extracts from bacterial lysate not transformed with TRβ1 expression vectors were incubated with the above DNA framents, producing a group of nonspecific protein-DNA complexes that was distinct from the binding site of TR (Fig. 7, lane 1). The addition of RXRβ increased the binding affinity of TR with this probe (Fig. 7, lane 3) The specificity of the interaction between TR and the pro-αl(I) collagen gene TRE was determined by competition studies. The unlabeled restriction fragment at position −35–+115 eliminated the binding of the human TRβ1 to the labeled probe when present at up to 20-fold molar excess (Fig. 7, lanes 4 and 5). In contrast, the non specific DNA did not compete binding to the −35–+115 collagen gene 5′-flanking region when added in a 20-fold molar excess (Fig. 7, lane 6). The results from the TR-DNA binding assays demonstrated the binding of the human TRβ1 to a fragment containing a TRE located between position −35 and +115 in the 5′-flanking region of the rat pro-α1(I) collagen gene.

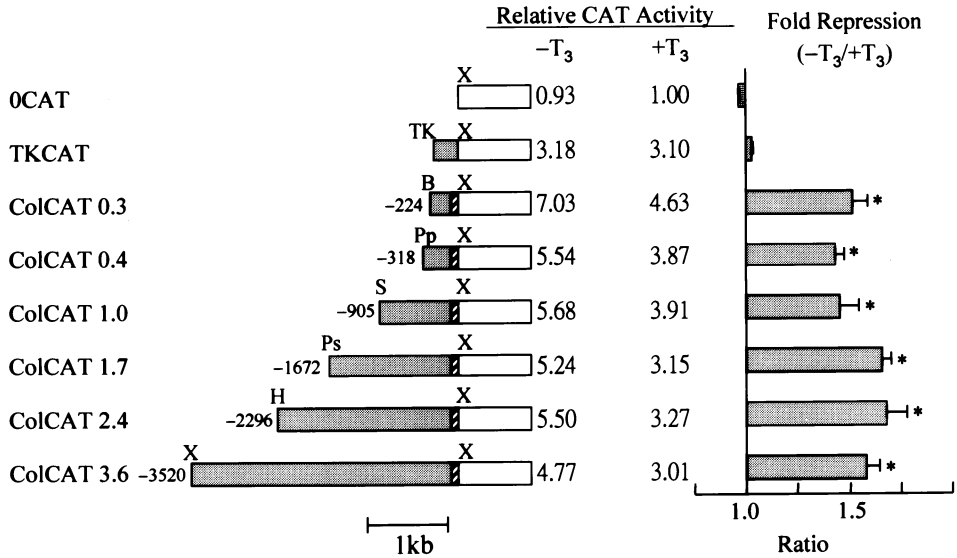

Fig. 6. Identification of the pro-α1(I) collagen gene 5′-flanking region mediating negative regulation by T_3. A schematic linear map of ColCAT 3.6 and the 5′ end points of the deletion constructs are shown on the left. Solid bars represent the pro-α1(I) collagen gene 5′-flanking region in the construct, hatched bars represent the 115 bases of first exon and open bars represent the CAT gene with the SV 40 polyadenylation site. Restriction sites are indicated as follows: X, *Xba*I; B, *Bgl*II; Pp, *PpuM*I; S, *Stu*I; Ps, *Pst*I and H, *Hind*III. TK indicates thymidine kinase promoter. Constructs are named by the number of base pairs in the 5′ flanking region of the pro-α1(I) collagen gene. Cos-1 cells were co-transfected with the indicated ColCAT constructs (1 μg) containing various lengths of promoter sequences and TRβ1 (l μg). CAT activity was assayed as described in Section 2. The relative CAT activity is shown relative to the CAT activity of the promoterless plasmid p0CAT with stimulation by T_3. The ratio of the unstimulated divided by the stimulated T_3 radioactivity is plotted as a bar graph at the right-hand side of the Figure. The values shown represent the average ± S.D. of three separate transfections with each plasmid, each assayed in triplicate. * indicates a difference from the p0CAT at 0.05 significant level.

4. Discussion

The effect of thyroid hormone on collagen metabolism and the fate of myocardial collagen matrix in thyroid hormone-induced ventricular hypertrophy have been previously investigated. Yao and Eghbali (1992) demonstrated a decreased biosynthesis of type I collagen at the level of mRNA and protein in thyroxine-induced ventricular hypertrophy. They also found that the expression of type I collagen gene is up-regulated in the ventricular myocardium of thyroidectomized rats (Klein et al., 1996). Together, these findings suggest that the collagen matrix of the heart is responsive to the effects of thyroid hormone. In this study, we employed rat cardiac fibroblasts cultured in defined conditions to investigate the molecular mechanisms of myocardial fibrosis during hypothyroidism. The species was selected because of the availability of a well characterized culture system for studying the regulation of type I collagen. The results obtained from our in vitro model system are consistent with previous in vivo findings demonstrating the up-regulation of type I collagen gene expression in thyroid hormone-depleted conditions.

Many factors, including hemodynamic overload and humoral factors, have been reported to induce abnormal proliferation of cardiac fibroblasts and deposition of the collagen (Carver et al., 1991; Sadoshima et al., 1992; Sadoshima and Izumo, 1993; Schorb et al., 1993; Crabos et al., 1994). However, it has been suggested that proliferation of cardiac fibroblasts and increased collagen gene expression may not be concurrent events

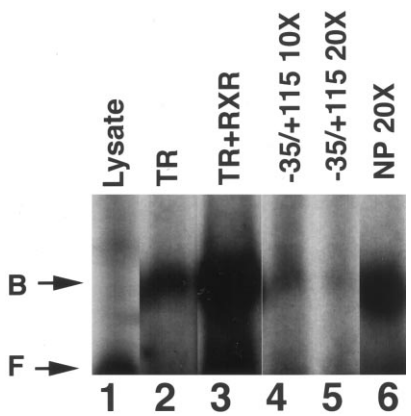

Fig. 7. Competition gel shift binding assay of TR to fragments containing the pro-α1(I) collagen gene 5′-flanking regions. Competition binding assays using a purified TR and one 5′-flanking DNA fragment (− 35– + 115) were performed as described in Section 2. ^{32}P-labeled DNA fragments were resolved by polyacrylamide gel electrophoresis in the presence of extracts from bacterial lysate not transformed with TR expression vectors (lane 1), the purified TR (1 μg) (lane 2) and the TR/RXR (lane 3). One microgram of the purified TR was preincubated with the unlabeled − 35– + 115 fragment (lanes 4 and 5) and unlabeled non-specific (NP) DNA(lane 6). B, bound TR-DNA complexes; F, free DNA.

in some models of myocardial fibrosis, these processes are under distinct regulatory control (Eghbali et al., 1991; Yao and Eghbali, 1992). In our study, rat cardiac fibroblasts were found to increase in the proliferation and biosynthesis of fibrillar collagen under thyroid hormone depleted conditions. Our findings conflict with previous studies using rabbit cardiac fibroblasts which indicated that exogeneous adminstration of thyroid hormone stimulates cellular proliferation (Yao and Eghbali, 1992). The disparity of these results may be due to the use of different preparations of thyroid hormone and differences in study designs. Additionally, there is convincing evidence that the effects of thyroid hormone are species-dependent (Crozatier et al., 1991).

It is recognized that fibrosis is caused by excess deposition of fibrillar collagens due to an imbalance between the collagen production and degradation processes in the existing cardiac fibroblasts. Our results showed that in rat cardiac fibroblasts, hypothyroidism, in addition to stimulation of proliferative response, enhances type I collagen biosynthesis at the levels of mRNA and protein. Therefore, a combination of both events may contribute to the development of observed myocardial fibrosis. On the other hand, although in this study the effects of hypothyroidism on collagenase gene expression and activity were not determined, the results of other studies suggest that collagenase, the key enzyme for collagen degradation, may be regulated by thyroid hormone (Karim et al., 1991; Lopez-Barahona et al., 1995). The regulatory effect of thyroid hormone on collagenase genes expression merits further investigations.

In this study, the chimeric ColCAT gene constructs were transiently expressed in primary rat cardiac fibroblasts and monkey COS-1 cells. It is well known that TR is present at very low level, even in highly responsive cell types (Brent et al., 1991). Therefore, either in cells with (cardiac fibroblasts) or without TR (COS-1 cells), the chimeric ColCAT plasmids should be simultaneously cotransfected with the TR expression plasmid to examine T_3-dependent repression. Similar results were obtained using these two types of cells. Since most studies of the regulation of collagen genes have been performed in fibroblast cell lines, the relative activities of promoter in other tissues or cell types have not been well defined. Tissue specificity represents an interaction between DNA-binding proteins, which may be expressed or active only in certain cell types and *cis*-elements within each tissue-specific gene (Slack et al., 1993). Thus, whether the results of our study obtained from mesenchymal-like cells can be applied to other cell types needs to be further clarified.

In this study, we have functionally mapped the 5′-flanking region of the rat pro-α1(I), collagen gene which is important in mediating basal and regulated expression by T_3. Deletion analysis of the pro-α1(I) collagen gene showed that the constructs containing $-224-+115$ bp of the 5′-flanking sequence maintains the trans-activation by TR. However, this responsiveness is ablated by deletion of this region. These results localize the region that may contain a functional TRE to the $-224-+115$ bp region.

To demonstrate the binding of TR in this region, we performed the TR-DNA shift binding assays using DNA fragments corresponding to two regions of the 5′-flanking sequence of the pro-α1(I) collagen gene. The binding studies confirmed that the human TRβ1 can bind to the -35 and $+115$ bp region specifically and with high affinity. The 5′-flanking region of the rat pro-α1(I) collagen gene is GC-rich. This region contains a TATA box at position -28 bp and a reverse CAT box at position –100 bp. Computer-assisted analysis of the $-35-+115$ pro-αl(I)collagen genomic region shows an 86% homology with the binding sequence of v-*erb*-*A* in the $+48-+68$ bp region (Sap et al., 1989). However, a palindromic sequence characteristic of many thyroid hormone-response genes is not present.

A recent study has demonstrated that an activator protein-1 (AP-1) ($+92-+97$) response element on the pro-α1(I) collagen gene is necessary for thyroid hormone-induced inhibition of promoter activity in cardiac fibroblasts. Nuclear proteins of T_3-treated cardiac fibroblasts binded to the AP-1 response element specifically (Lee et al., 1998). In agreement with this study, our binding studies demonstrate that the purified TR can bind to the -35 and $+115$ bp region containing the AP-1 response element. Together, these findings confirmed the cross-talk between various signal transduction pathways involving nuclear receptors and members of the leucine zipper transcription factor family, such as AP-1, in regulating the pro-α1 (I) collagen gene expression (Yang-Yen et al., 1990; Zhang et al., 1991).

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