

# Decreased Collagen Gene Expression and Absence of Fibrosis in Thyroid Hormone-Induced Myocardial Hypertrophy

## Response of Cardiac Fibroblasts to Thyroid Hormone In Vitro

Jianling Yao and Mahboubeh Eghbali

The regulatory effects of thyroid hormone on biosynthesis of myocardial proteins that originate from cardiac myocytes are well established. Little is known, however, of regulatory effects of thyroid hormone on interstitial proteins. In this study we examined the effects of thyroid hormone on collagen gene expression in thyroid hormone-induced myocardial hypertrophy and the response of cardiac fibroblasts to thyroid hormone in culture. Adult male Sprague-Dawley rats were treated intraperitoneally with L-thyroxin (10 µg/100 g body wt) for 2 hours or 1, 2, 3, 6, 12, or 14 days. Northern blot analysis of RNA from total ventricular tissue showed that after 2 hours of treatment, the abundance of mRNA for pro  $\alpha_2(I)$  collagen decreased by 53% ( $p < 0.05$ ) and reached the lowest level (60% decrease,  $p < 0.02$ ) at day 1, remained diminished at day 3, and then gradually returned toward normal levels. After transient transfection of chimeric DNA containing collagen type I promoter-chloramphenicol acetyl transferase (CAT) gene into the thyroxin-treated cardiac fibroblasts, the level of CAT activity decreased significantly. Treatment of cardiac fibroblasts in culture (10 nM L-thyroxin) resulted in a 33% ( $p < 0.005$ ) decrease in the abundance of mRNA for pro  $\alpha_2(I)$  collagen. The stability of the mRNA for pro  $\alpha_2(I)$  collagen in cardiac fibroblasts, as measured by mRNA half-life, was slightly (16.6%) decreased by thyroid-hormone treatment. Collagen synthesis as shown by immunofluorescent staining of intracellular collagen in cultured fibroblasts and in frozen sections of myocardium was also diminished. Interestingly, the abundance of mRNA for transforming growth factor- $\beta_1$  in the myocardium increased by 53% ( $p < 0.05$ ) within hours and reached a peak (75% increase,  $p < 0.001$ ) at day 1 after treatment. The abundance of mRNA for transforming growth factor- $\beta_1$  was also increased (94%,  $p < 0.005$ ) in cardiac fibroblasts after treatment with L-thyroxin. Thyroxin treatment of cardiac fibroblasts led to the induction of proto-oncogenes *c-fos* and *c-jun* and early growth response gene within 1 hour. Treatment of cardiac fibroblasts with L-thyroxin led to a 357% ( $p < 0.001$ ) increase in [<sup>3</sup>H]thymidine incorporation into the cell nuclei compared with that in untreated cells. Together, these findings suggest that cardiac fibroblasts and proteins originating from them are targets for thyroid hormone action in the myocardium and that this hormone plays a regulatory role on interstitial protein gene expression. Data further suggest that thyroid hormone-induced myocardial hypertrophy could be distinguished by the lack of cardiac fibrosis. (Circulation Research 1992;71:831-839)

**KEY WORDS • extracellular matrix • endocrine • myocardium • hypertrophy • gene expression • thyroid hormone • DNA transfection**

**R**egulatory effects of thyroid hormone on cardiac myocytes and on contractile protein gene expression in the heart have been well established.<sup>1-3</sup> Little is known, however, about the effect of this hormone on cardiac interstitium and interstitial protein gene expression.<sup>3</sup> Although effects of thyroid hormone on collagen biosynthesis<sup>4</sup> and the wound heal-

ing process<sup>5</sup> have been observed for a long time, the direct effect of thyroid hormone on gene expression for various collagen types in general and its effect on cardiac tissue with regard to collagen gene expression in particular is unknown. It is now established that collagen matrix of the heart plays an important role in maintaining the integrity of myocardial function. Collagen type I is the major component of collagen matrix in the heart and originates from cardiac fibroblasts.<sup>6</sup> In previous studies, we have shown that myocardial hypertrophy, induced by pathological and physiological conditions such as pressure overload and normal growth and aging, is accompanied by cardiac fibrosis and that those conditions of hypertrophy cause quantitative and qualitative changes in the biosynthesis and deposition of the myocardial collagen matrix.<sup>7-9</sup> In this study, we have

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From the Cardiovascular Institute, Michael Reese Hospital, Department of Medicine, University of Chicago, Pritzker School of Medicine.

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Address for reprints: Mahboubeh Eghbali, PharmD, PhD, Department of Anesthesiology, Yale University School of Medicine, 333 Cedar Street, PO Box 3333, New Haven, CT 06510.

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examined collagen gene expression in the rat model of myocardial hypertrophy induced by thyroid hormone and examined the response of cardiac fibroblasts to thyroid hormone in culture. The results provide insights into the mechanism of action of thyroid hormone with regards to myocardial collagen biosynthesis. They also provide information that although related to the heart tissue, may prove to be the reflection of the general regulatory role of thyroid hormone on collagen gene expression in various tissues.

### Materials and Methods

#### *Animal Model*

Adult male Sprague-Dawley rats (200–220 g) were maintained on standard rat chow diet with access to water and received daily injections (10 µg/100 g body wt i.p.) of L-thyroxin. A total of 100 rats were used for all steps involving initial studies for dose determination, blood pressure measurement, data collection, and immunofluorescent studies. All survived the full course of treatment. Rats were killed at an early stage (within hours) or over an extended period of 14 days after treatment (days 1, 2, 3, 6, or 12 for mRNA studies and day 14 for immunofluorescent light microscopy). Mean arterial pressure, body weight, and ventricular heart weight were measured at the end of each time point.

#### *Preparation and Treatment of Cardiac Fibroblasts in Culture*

Cardiac fibroblasts were prepared as previously described.<sup>10</sup> Briefly, adult male New Zealand White rabbits were anesthetized, and hearts were excised, minced, and washed in 1× phosphate buffered saline (PBS). The tissue was then subjected to digestion at 35°C by a mixture of 0.1% trypsin and 200 IU/ml collagenase (type IV, Sigma) for 10 minutes by constant shaking in a 25-ml glass Erlenmeyer flask. Isolated cells were pelleted at the end of 10-minute digestion periods and plated on 100-mm culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were incubated with 90% O<sub>2</sub>+10% CO<sub>2</sub> for 2 hours at 37°C in an incubator with a humidifier. The unattached cells were then discarded, and attached cells (mostly fibroblasts) were grown in DMEM plus 10% FBS. The fibroblastic nature of the cells was determined by immunofluorescence staining with anti-human factor VIII for detection of endothelial cells, anti-desmin for muscle cells, and anti-vimentin for fibroblasts, as previously described.<sup>10</sup> Confluent cells were either passaged or stored. For all studies, cells from passages 4–9 were used. For drug treatment, cells were grown until confluent in DMEM plus 10% FBS on 100- or 60-mm culture dishes. They were then serum deprived (0.5–1%) for 24 hours. L-Thyroxin (10 nM) was added to the culture medium. Treatment lasted for varying amounts of time. For studies involving mRNA half-life, cells were treated with L-thyroxin for 24 hours and RNA synthesis was inhibited by 5,6-dichloro-L-β-D-ribofuranosyl benzimidazole (DRB).<sup>11</sup> This substance is the inhibitor of transcription initiation and was added to the medium at 60 µM.

#### *Transient DNA Transfection*

Cardiac fibroblasts were seeded at a density of 0.25×10<sup>6</sup> per 60-mm dish 16 hours before transfection.

Six micrograms of plasmid DNA containing the 3.6 kb of upstream rat  $\alpha_1(I)$  collagen promoter that was ligated into the *Xba* site of a chloramphenicol acetyl transferase (CAT) gene<sup>12</sup> (generous gift of Dr. David Rowe) was mixed with 20 µg lipofectin (1 mg/ml solution; BRL, Gaithersburg, Md.) according to the manufacturer's guidelines and added to the cell medium. Before the addition of DNA-lipofectin complex, cell layers were washed with and maintained in serum-free medium. Twelve hours later, lipofectin was removed, and the cells were treated for 4–6 hours with medium containing 10% serum. Cells were then incubated for 48 hours in serum-free (0.5–1%) medium in the presence of 10 nM thyroxin. Control cells in identical conditions were transfected with equal amounts of plasmid DNA and received equal aliquots of vehicle (0.1N NaOH) instead of thyroxin. To examine nonspecific effect of thyroxin on promoter activity, parallel groups of cells were transiently transfected with 6 µg plasmid DNA containing SV40 promoter sequence linked to CAT reporter gene. Cells were treated under identical conditions as those that received  $\alpha_1(I)$  collagen promoter-CAT constructs.

#### *Determination of the Activity of $\alpha_1(I)$ Collagen Promoter*

The activity of  $\alpha_1(I)$  collagen and SV40 promoter in thyroxin-treated and control (untreated) cardiac fibroblasts was determined by measuring CAT enzyme activity according to Gorman et al.<sup>13</sup> In each assay, aliquots of cell lysate containing equal amounts of protein from thyroxin-treated and control cells were assayed.

#### *RNA Extraction*

For studies of cardiac tissue, total RNA was extracted by the procedure of Chirgwin et al,<sup>14</sup> with minor modifications as previously described.<sup>8</sup> Briefly, hearts were rapidly dissected, atria removed, and ventricular myocardium homogenized in 4 M guanidine thiocyanate solution on ice. The homogenate was cleared of cell debris by centrifugation at 10,000g for 10 minutes. RNA was pelleted on a cesium chloride cushion and then redissolved in Tris-EDTA (pH 7.4) and purified by three ethanol precipitations. Total RNA was quantified by absorbance at 260 nm, assuming 40 µg/ml for each unit of absorbance.

For studies of cardiac fibroblasts, RNA was extracted from confluent cell layers in 100-mm culture dishes by modification of the method of Chomczynski and Sacchi.<sup>15</sup> Briefly, cell layers were washed twice with PBS, 4 M guanidine isothiocyanate was added to the dishes, and cells were lysed by scraping and vortexing. Equal volumes of phenol (pH 7.4) were added to the cell lysate, and the mixture was centrifuged in a Sorvall centrifuge at 10,000g at 4°C for 20 minutes. The aqueous phase containing the RNA was then transferred to a fresh tube, and RNA was prepared by two steps of precipitation with 70% ethanol.

#### *RNA-DNA Hybridization*

Steady-state levels of mRNA were determined by Northern hybridization analysis. Ten micrograms of total RNA was denatured in 50% formamide, 17.5% formaldehyde, and 1× MOPS buffer (20 mM 3-[*N*-morpholi-

no]propanesulfonic acid at pH 7.0, 5 mM sodium acetate, and 1 mM Na<sub>2</sub>-EDTA at pH 8.0), electrophoresed in a 1% agarose gel, transferred to a Gene-Screen Filter (New England Nuclear, Boston), and baked for 2 hours at 80°C in a vacuum oven. Blots were prehybridized in 5× SSC, 0.1% SDS, 5× Dehnardt's solution, 50% deionized formamide, 0.1 M phosphate buffer (pH 7.0), and 500 µg/ml calf thymus DNA for 24 hours at 42°C with the same buffer containing the appropriate radioactive probes to obtain 3×10<sup>6</sup> counts/min per milliliter of hybridization medium. The cDNAs were radioactively labeled by random primer extension, as described by Feinberg and Vogelstein,<sup>16</sup> through the use of Amersham Multiprime DNA labeling systems according to the manufacturer's manual (Amersham, UK). [<sup>32</sup>P]-dCTP (specific activity, 300 Ci/mM; Amersham) was included in the reaction mixture to obtain a specific activity of 2–6×10<sup>8</sup> counts/min per microgram DNA. Recombinant plasmids used as probes were as follows: rat α<sub>2</sub>(I) sequences specific for the entire 3' noncoding and C-terminal propeptide regions that hybridize to 4.2 and 4.5 kb mRNA<sup>17</sup>; a full length cDNA for chicken β-actin<sup>18</sup>; a cDNA probe of approximately 1,050 bp to human transforming growth factor-β<sub>1</sub> (TGF-β<sub>1</sub>)<sup>19</sup>; full length cDNA probes to rat c-fos and rat c-jun<sup>20</sup>; and a 2.0-kb EcoRI fragment of the mouse early growth response gene (Egr-1) cDNA clone OC68.<sup>21</sup> After hybridization, the membranes were washed and exposed to Kodak XAR-5 film at –70°C. Prehybridization, hybridization, and washing procedures were performed according to the methods previously described.<sup>8,9</sup>

**Immunofluorescent staining.** Cardiac fibroblasts were cultured in DMEM plus 10% FBS on Lab-Tek Permanox chamber slides or in 60-mm culture dishes (NUNC, Inc.) until confluent. They were then deprived of serum (0.5–1%) for 24 hours. L-Thyroxin (10 nM) was added to the culture over a 72-hour period. Control dishes received an equivalent volume of vehicle (0.1N NaOH). At the end of treatment, cell layers were washed twice with PBS and permeabilized in absolute methanol for 7 minutes at 4°C. For myocardial tissue studies, rats were treated for 14 days with L-thyroxin (10 µg/100 g body wt). Preparation of frozen sections (12 µm thick) was performed as previously described.<sup>9</sup> Staining of cell layers and tissue sections was performed as previously described.<sup>9</sup> Briefly, samples were layered with 1:50 dilution of a rabbit anti-rat tail tendon type I collagen antibody,<sup>8,9</sup> incubated at room temperature for 1 hour, and washed four times with PBS. Each wash lasted 2 minutes. As negative controls, parallel slides were incubated with immunoglobulin G (IgG) prepared from rabbit preimmune serum. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used as second antibody. Visualization was performed with an Olympus model BH<sub>2</sub> epifluorescent microscope. Tri-X-400 film with 2 minutes' exposure time was used for photography.

**<sup>3</sup>H]Thymidine incorporation.** Cardiac fibroblasts were grown until confluent in DMEM plus 10% FBS on 35-mm culture dishes. They were then serum deprived (0.5–1%) for 24 hours. L-Thyroxin (10 nM) was added to the culture medium for 22 hours. [<sup>3</sup>H]Thymidine (5 µCi/ml) (specific activity, 86 Ci/mmol [Amersham]) was added to the medium. After 3 hours, cells were washed three times with PBS and lysed in 0.1% sodium dodecyl

sulfate and 0.1 M NaOH. DNA was precipitated by 5% trichloroacetic acid. The precipitate was filtered, and then the filters were washed with 10% trichloroacetic acid and 95% ethanol. The radioactivity of the filters was determined by liquid scintillation counting.<sup>10</sup> DNA was quantified by the use of chromogenic reagent (0.1 M acetaldehyde, 1.5% diphenylamine in glacial acetic acid, and concentrated sulfuric acid).

#### Quantification and Data Analysis

mRNAs and promoter activity were quantified by densitometry scanning (Hoefer) of Northern and CAT assay autoradiographs, respectively. To determine CAT activity, the density of acetylated [<sup>14</sup>C]chloramphenicol and untransformed chloramphenicol spots on the autoradiogram was measured. CAT activity was the percentage of acetylated [<sup>14</sup>C]chloramphenicol in a 1-hour reaction. To determine the abundance of mRNA, ribosomal RNA bands on the gene screen membrane were visualized by ethidium bromide staining and photographed. The density of the 28S ribosomal RNA band was measured by densitometry scanning of the negatives. To obtain a precise measurement of mRNA per unit total RNA (i.e., normalized mRNA), the density of an individual mRNA band was divided by the density of the corresponding 28S ribosomal RNA band on the gene-screen membrane. To eliminate intergel density differences, all data are presented as a percent of the control value (normalized mRNA for treated group/normalized mRNA for the corresponding control group). It should be noted that samples from a treated group and the corresponding controls were always run on a single gel.

As discussed above, the mRNA measurement, presented as a percent of control, represents the ratio of two means (i.e., treated group mean/control group mean). Standard error of this percent mRNA was computed from the standard errors of measured data using the principle of propagation of error.<sup>22</sup> The percent mRNA data at different times were compared with controls (i.e., 100%) by computing the *t* statistics, with the significance level adjusted for multiple comparisons by Bonferroni bounds.

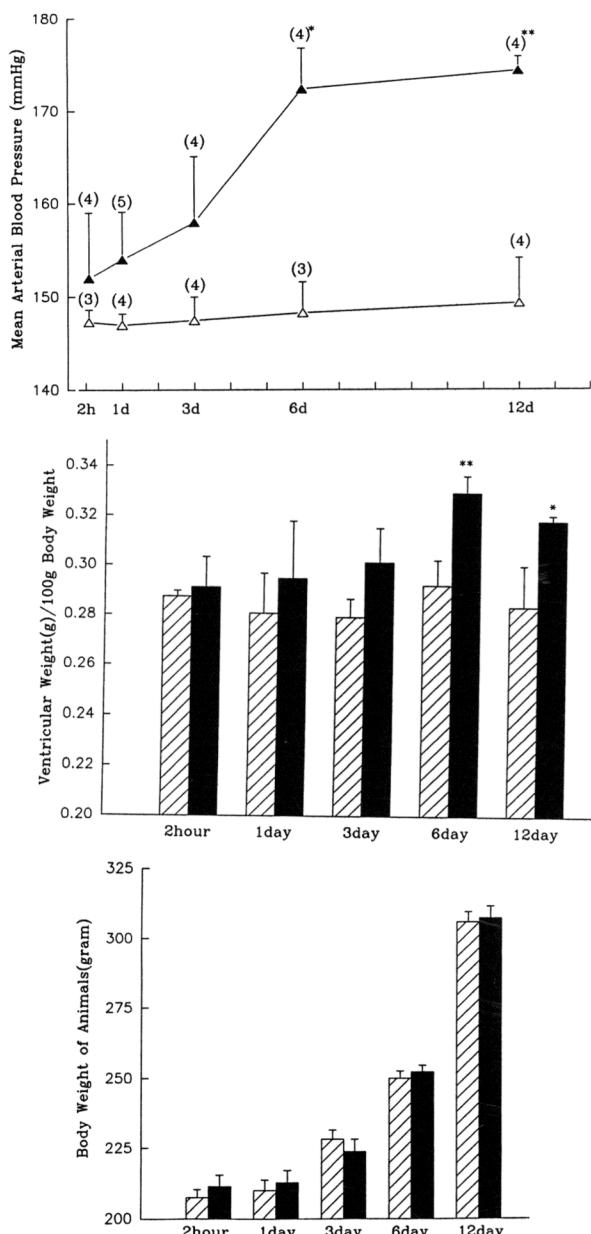
## Results

### Blood Pressure and Heart Weight

Treatment of rats with L-thyroxin (10 µg/100 g body wt) resulted in an increased mean arterial pressure that was statistically significant after 6 days of treatment (Figure 1). The induction of myocardial hypertrophy was examined by comparing the ratio of total ventricular weight to the body weight in thyroxin-treated and control rats over 12 days of treatment (Figure 1). In the treated group this ratio began to increase from day 1, and at day 6 it was increased by 13% (*p*<0.01). The increase in the ratio of ventricular weight to body weight was caused by the increased ventricular weight and not the reduced body weight values (Figure 1).

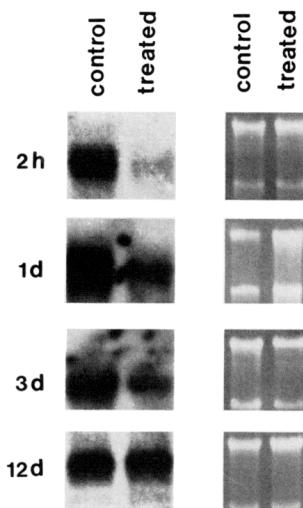
### Steady-State Levels of mRNA

**Ventricular tissue.** To examine the regulatory effects of thyroid hormone on fibrillar collagen gene expression, we measured the abundance of mRNA for pro α<sub>2</sub>(I) collagen within hours (early stage) and over an



**FIGURE 1.** *Blood pressure and myocardial hypertrophy.* Adult male Sprague-Dawley rats (200–220 g) received daily injections (10  $\mu$ g/100 g i.p.) of L-thyroxin. Rats were killed at indicated times after treatment ( $\blacktriangle$ — $\blacktriangle$ ). For each group, age-matched untreated rats were used as control ( $\triangle$ — $\triangle$ ). Mean arterial pressure was measured by intra-arterial cannulation. Body weight and heart weight were measured at the end of each time point. Data are expressed as mean  $\pm$  SD ( $n=4$  or 5 in each group). \* $p<0.05$ , \*\* $p<0.01$ .

extended period of 12 days. The results of Northern hybridization analysis of RNA extracted from ventricular tissue demonstrated that the abundance of mRNA for pro  $\alpha_2(I)$  collagen in treated hearts decreased by 53% ( $p<0.05$ ) within hours, reached its lowest level (60% decrease,  $p<0.02$ ) at day 1, remained diminished until day 6, and gradually returned to control levels at day 12 (Figure 2). We also measured the abundance of mRNA for TGF- $\beta_1$ , a regulator of collagen type I gene expression. Interestingly, the abundance of mRNA for



**FIGURE 2.** *Northern hybridization analysis of ventricular RNA for pro  $\alpha_2(I)$  collagen.* RNA was extracted from total ventricular myocardium of the thyroxin-treated rats and control age-matched untreated rats. Electrophoresis, transfer of RNA, and washing procedures were as described in "Materials and Methods." Left panel: mRNA for pro  $\alpha_2(I)$  collagen from treated and untreated hearts. Right panel: Corresponding ribosomal RNA bands as visualized by ethidium bromide staining. These autoradiographs are representatives of three individual experiments.

TGF- $\beta_1$  in the ventricular myocardium of treated hearts increased at early stage by 53% ( $p<0.05$ ) and reached a peak (75%,  $p<0.001$ ) at day 1 after treatment. The abundance of mRNA for TGF- $\beta_1$  declined toward those in control hearts thereafter (Figure 3). To test the effect of thyroid hormone on cytoskeletal protein gene expression, we also measured the abundance of mRNA for cytoskeletal actin. Within hours of L-thyroxin treatment there was a 47% ( $p<0.05$ ) decrease in the abundance of mRNA for cytoskeletal actin that persisted through day 3. The mRNA levels returned to control levels at day 12 (Figure 3).

**Cardiac fibroblasts.** Since cardiac fibroblasts are the cellular origin of collagen in the heart, we measured the abundance of mRNA for pro  $\alpha_2(I)$  collagen and cytoskeletal actin in cardiac fibroblasts over a 72-hour treatment with L-thyroxin to gain insights into the effect of thyroid hormone on these cells. We also measured the abundance of mRNA for TGF- $\beta_1$ . The results of Northern hybridization showed that at 24 hours the abundance of mRNA for pro  $\alpha_2(I)$  collagen in thyroxin-treated cells decreased by 33% compared with that in untreated cells (Figure 4). At 72 hours, mRNA abundance for pro  $\alpha_2(I)$  collagen remained diminished (34%,  $p<0.005$ ). Cytoskeletal actin mRNA levels were also decreased in thyroxin-treated cells by 28% ( $p<0.05$ ) at 24 hours and 39% ( $p<0.01$ ) at 72 hours. In contrast, TGF- $\beta_1$  mRNA levels in treated cells increased by 94% ( $p<0.005$ ) at 24 hours compared with those in untreated cells (Figure 4). The increased mRNA level declined thereafter but remained elevated (41% [ $p<0.05$ ]) at 72 hours of treatment.

Thyroxin treatment of cardiac fibroblasts also led to the induction of an early response. mRNAs for proto-

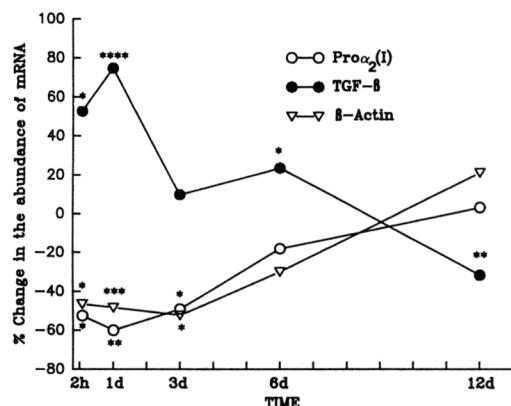


FIGURE 3. Results of densitometry scanning of autoradiograms from Northern hybridization analysis of total RNA from ventricular myocardium of rats treated with L-thyroxin and control age-matched rats. RNA (10  $\mu$ g) from treated and untreated ventricular tissues was applied to 1% agarose gel. Electrophoresis and blotting were performed as described in "Materials and Methods." Blots were hybridized to  $^{32}$ P-labeled cDNA probes to rat pro  $\alpha_2$ (I) collagen, human transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), and chicken  $\beta$ -actin. Densitometry scanning was performed to measure the density of mRNA and the corresponding 28S ribosomal RNA bands as described in "Materials and Methods." Data are expressed as means of three individual experiments. \* $p<0.05$ , \*\* $p<0.02$ , \*\*\* $p<0.01$ , \*\*\*\* $p<0.001$ .

oncogenes *c-fos*, *c-jun*, and *Egr-1* were induced in cardiac fibroblasts as early as 30 minutes after the addition of thyroxin to the culture medium (Figure 4).

#### Effect of Thyroid Hormone on Collagen Type I Promoter Activity

Transient transfection of chimeric DNA that contained 3.6 kb upstream  $\alpha_1$ (I) collagen promoter linked to CAT gene into the cardiac fibroblasts showed that treatment of the cells with thyroxin after the transfection led to significant decrease in CAT activity in treated compared with untreated cells (Figure 5).

#### Effect of Thyroid Hormone on Stability of Collagen Type I mRNA

To understand if thyroid hormone has an impact on collagen type I mRNA stability, we measured the rate of mRNA degradation in the presence of an inhibitor of RNA synthesis, DRB. It was shown that 24-hour treatment of cells with thyroxin led to only a 16.6% decrease in collagen type I mRNA apparent half-life (Figure 6).

#### Collagen Synthesis

To evaluate if the inhibitory effect of thyroxin on collagen promoter activity and mRNA leads to changes in collagen type I synthesis, we examined intracellular collagen type I expression in cultured cardiac fibroblasts as well as patterns of collagen deposition in the myocardium by immunofluorescent staining of frozen sections of the heart and cultured cells with anti-collagen type I antibody. It was shown that despite the presence of myocardial hypertrophy in thyroxin-treated rats at 14 days of treatment, and contrary to what is usually seen in other models of hypertrophy, collagen deposition did

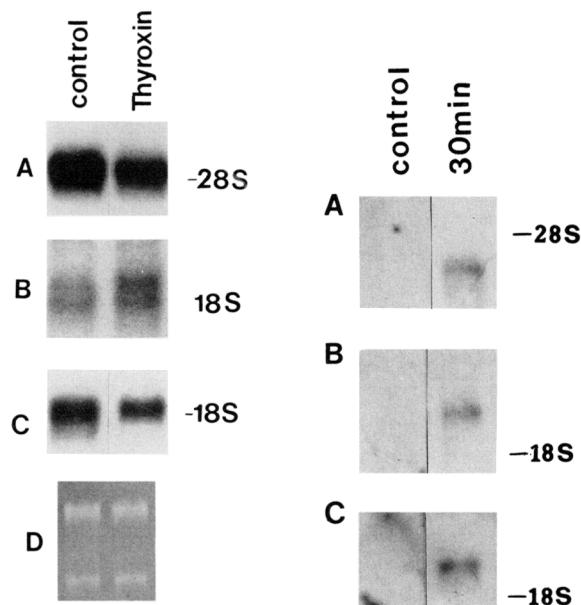


FIGURE 4. Autoradiograms of Northern hybridization analysis of total RNA from cardiac fibroblasts in culture. Ten micrograms RNA was subjected to size fractionation by electrophoresis on 1% agarose gel. RNA transfer and blot hybridization are described in "Materials and Methods." In the left panel, A shows changes in mRNA for pro  $\alpha_2$ (I) collagen following 24-hour treatment with L-thyroxin; B shows changes in transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) mRNA; C shows changes in mRNA levels for cytoskeletal actin; and D shows corresponding ribosomal RNA bands. In the right panel, A, B, and C show Egr-1, *c-fos*, and *c-jun* mRNAs, respectively. These autoradiograms are representatives of three individual experiments.

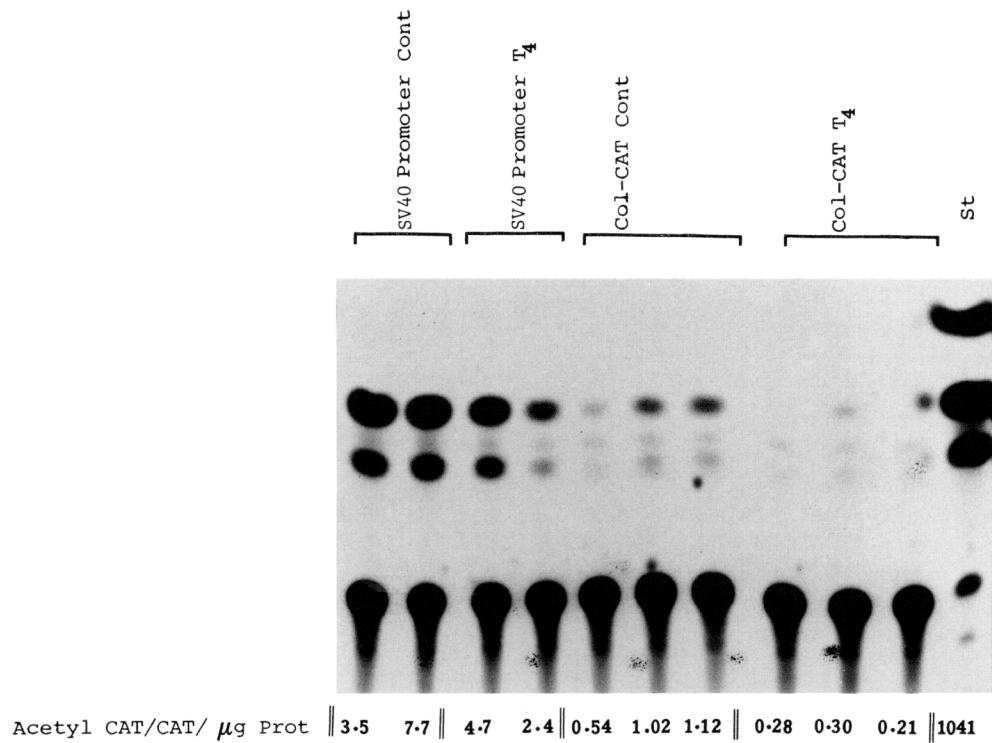
not increase, and the patterns of immunofluorescent staining in treated hearts were somewhat similar to those in untreated hearts (Figure 7). In cardiac fibroblasts, immunofluorescent staining of thyroxin-treated cells was diminished compared with that of untreated cells (Figure 7). It is also noteworthy that the morphology of treated cells as examined by phase contrast light microscopy showed no apparent differences with those of untreated cells.

#### Fibroblast Proliferation

To understand if thyroid hormone may have an impact on cardiac fibroblast proliferation, cultured cardiac fibroblasts were treated with L-thyroxin for 24 hours, and their capacity to proliferate was determined by the measurement of [ $^3$ H]thymidine incorporation into the cell nuclei. The results showed that thyroxin treatment of the confluent quiescent cells for 24 hours led to a 357% ( $p<0.001$ ) increase in [ $^3$ H]thymidine incorporation into the cell nuclei compared with that in untreated cells (Figure 8).

#### Discussion

Influence of thyroid hormone on cardiac myocytes and myocyte-specific proteins, such as  $\alpha$ - and  $\beta$ -myosin heavy chain, has been extensively investigated. Little is known, however, about the effect of thyroid hormone on expression of collagen gene in the myocardium and the

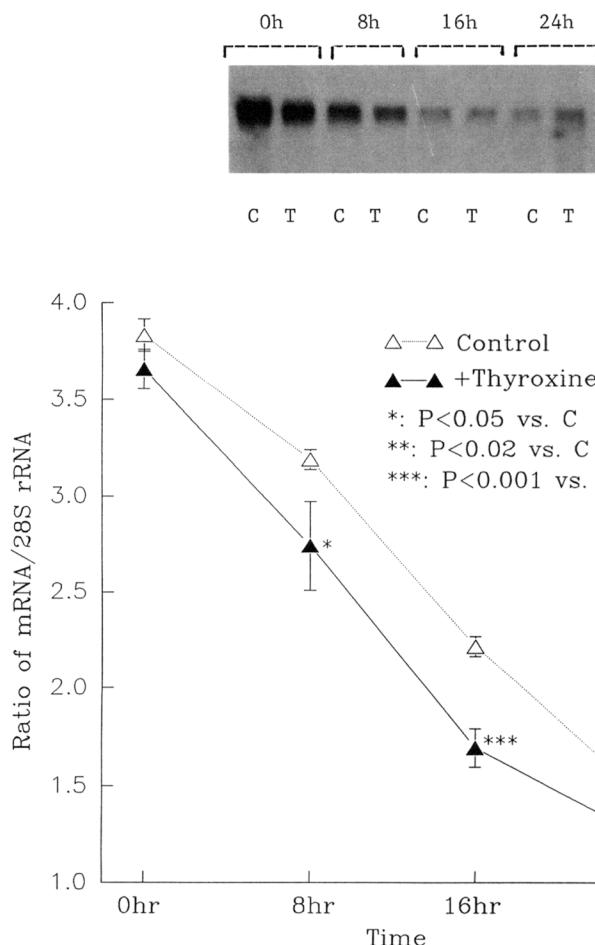


**FIGURE 5.** Effect of thyroid hormone on transfected collagen type I promoter in cardiac fibroblasts. Plasmid DNA containing 3.6 kb fragment of collagen type I promoter and CAT gene or SV40 promoter and CAT gene was transfected into the cardiac fibroblasts by lipid-DNA complex (lipofectin) procedure as described in "Materials and Methods." After the transfection, cells were treated with 10 nM L-thyroxin, and 48 hours later aliquots of cell lysate containing 0.3–0.35  $\mu$ g protein were analyzed for CAT activity. CAT activity was measured as the ratio of acetylated CAT to untransformed CAT per 1  $\mu$ g protein in 1-hour reaction.

responsiveness of interstitial cells to this hormone. Our findings have several significant aspects. 1) Abundance of mRNA for collagen type I, the major fibrillar collagen of the heart, is downregulated by thyroid hormone both in the myocardium and in cardiac fibroblasts in culture. This downregulation of mRNA leads to the inhibition of collagen synthesis as shown by the lack of myocardial fibrosis in the myocardium despite thyroid hormone induction of cardiac hypertrophy and by diminished intracellular collagen in cardiac fibroblasts. 2) Transient transfection experiments with collagen type I promoter-CAT construct and studies on mRNA half-life in cardiac fibroblasts suggest that inhibitory effect of thyroid hormone in vitro is most likely at the transcriptional level and only partially caused by the changes in mRNA stability. However, in vivo results regarding decline of mRNA abundance at early stages of treatment indicate that posttranscriptional regulation and/or secondary induced factors may be involved. 3) Since thyroid hormone treatment led to increased mRNA abundance for other proteins such as TGF- $\beta_1$ , its inhibitory effect on collagen biosynthesis is not caused by a general inhibition of gene expression. 4) Thyroid hormone treatment increases proliferation of cardiac fibroblasts in culture. Diminished abundance of mRNA for cytoskeletal actin showed that thyroid hormone treatment is not likely to induce cardiac fibroblast hypertrophy. Cardiac fibroblasts' intact morphology after treatment with thyroid hormone is an indication of the lack of cellular toxicity.

Regulation of myocardial collagen gene expression in rat models of cardiac hypertrophy induced by hemodynamic changes due to increased blood pressure has been

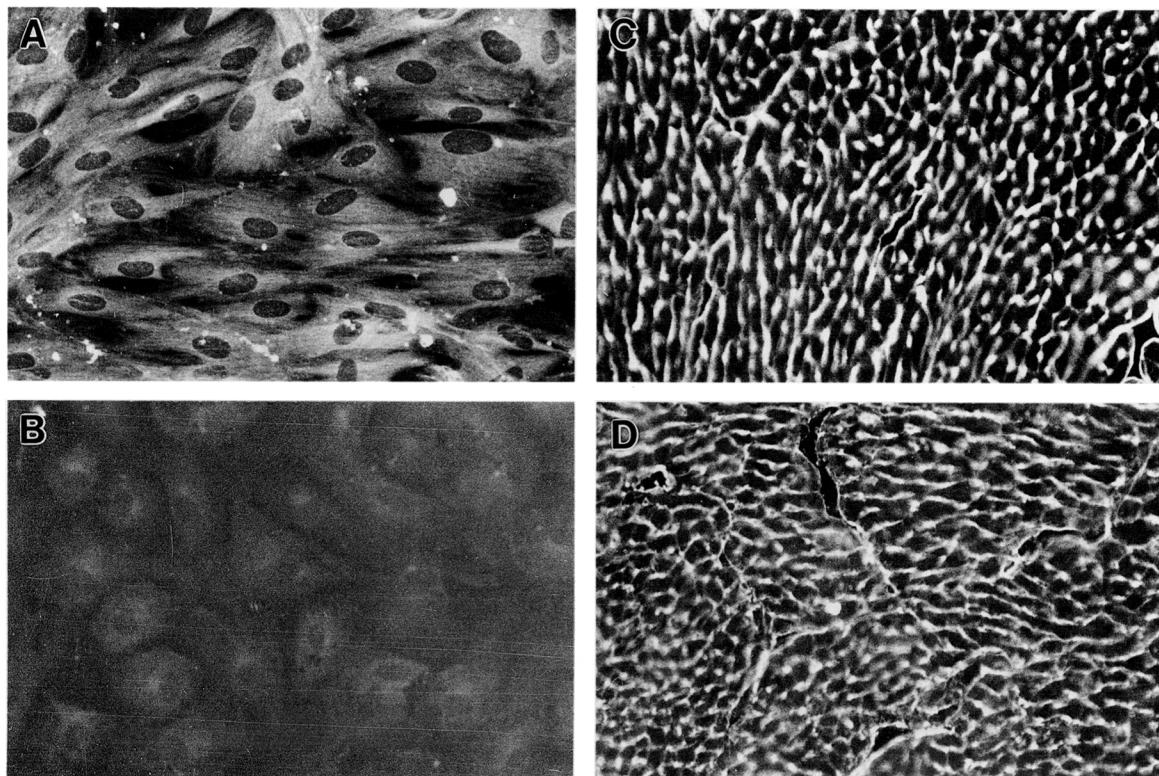
previously established.<sup>8,9</sup> In those studies we have demonstrated that increased blood pressure induced by abdominal aortic banding<sup>8</sup> and by intravenous infusion of norepinephrine<sup>9</sup> leads to increased mRNA levels for collagen type I and that cardiac fibrosis is an integral part of myocardial hypertrophy induced by the above interventions. In the present study, in the myocardium of thyroxin-treated rats, collagen deposition as shown by immunofluorescent light microscopy was not significantly different from that in control hearts. These findings are the first evidence of inhibition of myocardial collagen type I gene expression caused by hormonal regulation. Most importantly, they suggest that myocardial hypertrophy induced by thyroid hormone is the kind of cardiac hypertrophy that is not accompanied by cardiac fibrosis. These findings are particularly significant with regard to identification of various types of myocardial hypertrophy. Decreased abundance of mRNA for various proteins could be due to alterations in mRNA stability. It was shown that thyroid hormone had very little effect on the stability of mRNA for pro  $\alpha_2(I)$  collagen in cardiac fibroblasts in culture. However, decreased CAT activity in transient expression experiments after thyroxin treatment of transfected cultured cardiac fibroblasts indicates that thyroid hormone may indeed have an impact on the activity of collagen type I promoter and, hence, regulation of collagen gene expression at the level of transcription. Since nuclear protein factor has been identified in cardiac fibroblasts that bind to DNA fragments containing thyroid hormone-responsive element,<sup>23</sup> one may also speculate that the inhibitory effect of thyroid hormone



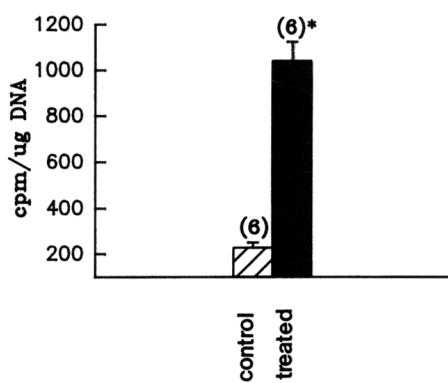
**FIGURE 6.** Stability of *pro α<sub>2</sub>(I)* mRNA in cultured cardiac fibroblasts. Confluent quiescent cardiac fibroblasts were treated with 10 nM L-thyroxin for 24 hours (T) or received equivalent volume of vehicle, 10 nM NaOH (C). At time 0, DRB, an inhibitor of RNA synthesis, was added at 60 μM. The abundance of mRNA for *pro α<sub>2</sub>(I)* collagen in control untreated and thyroid hormone-treated cells was measured on triplicate 100-mm dishes at indicated times. Upper panel: Representative autoradiogram of Northern hybridization analysis. Lower panel: Results of densitometry scanning of mRNA for *pro α<sub>2</sub>(I)* collagen. Data are expressed as mean ± SEM (n = 3 in each group).

on collagen type I gene expression is likely to be through direct effects of thyroid hormone on nuclear mechanisms; these effects are not dissimilar to those by which thyroid hormone is able to regulate myocardial myosin heavy chain gene expression. Alternatively, since *in vitro* thyroxin treatment led to the induction of transcription factors, i.e., proto-oncogenes *c-fos*, *c-jun*, and *Egr-1*, it is also likely that parts of the *in vivo* effects of thyroid hormone, especially those induced as an early response, are mediated indirectly through the induction of other hormones and factors. Another significant finding of the present investigation is that despite decreased expression of mRNA for *pro α<sub>2</sub>(I)* collagen, mRNA for TGF-β<sub>1</sub>, a regulator of collagen type I gene transcription, is upregulated by thyroid hormone both *in vivo* and *in vitro*. This indicates that thyroid hormone has a stimulatory effect on expression of TGF-β<sub>1</sub> mRNA, and it is very unlikely that this growth factor would be involved in the regulatory pathway by which thyroid hormone exerts its inhibitory effects on collagen gene expression. Increased fibroblast proliferation on the one hand and decreased abundance of mRNA for cytoskeletal actin and *pro α<sub>2</sub>(I)* collagen on the other, after L-thyroxin treatment, suggest that while thyroid hormone promotes the capacity of cardiac fibroblasts to proliferate, it specifically inhibits the expression of cytoskeletal protein and collagen type I genes and is not likely to induce cardiac fibroblast hypertrophy. Further studies involving actin biosynthesis and morphometric studies are needed to confirm the

effects of thyroid hormone on hypertrophy of cardiac fibroblasts. Data on cardiac fibroblast proliferation also provide further support for our previously stated idea that although cardiac fibroblast proliferation results in increased number of cells that manufacture collagen, it does not necessarily lead to enhanced collagen production in the myocardium. This is particularly true when a particular drug or hormone with stimulatory effect on cell proliferation causes specific inhibition of collagen gene expression in individual cardiac fibroblasts. Recently we have demonstrated that phorbol myristate acetate, a mitogenic agent, decreases collagen gene expression in cardiac fibroblasts. It was also shown that despite this inhibitory effect, phorbol myristate acetate increases the incorporation of [<sup>3</sup>H]thymidine into the nuclei of cardiac fibroblasts.<sup>24</sup> These findings may further imply that although in some models of cardiac fibrosis, such as those induced by pressure overload and norepinephrine infusion, cardiac fibroblast proliferation and increased collagen gene expression are concurrent events, they are under distinct regulatory mechanisms. Together, findings of this study suggest that similar to its effect on cardiac myocytes and on contractile apparatus of the heart, thyroid hormone has a major impact on cardiac fibroblasts and on the interstitial compartment of the myocardium. They further suggest that collagen type I is a thyroid hormone-sensitive protein. Interaction of thyroid hormone with collagen gene merits future investigations.



**FIGURE 7.** Immunofluorescent staining of cardiac fibroblasts and myocardial frozen sections with anti-type I collagen antibody. Confluent quiescent cardiac fibroblasts were incubated with L-thyroxin (10 nM) for 72 hours. Cell layers were then washed, permeabilized, and fixed as described in "Materials and Methods." For the study of myocardial tissue, rats were treated with L-thyroxin (10 µg/100 g body wt i.p.). Frozen sections 12 µm thick were cut in the cryostat as described in "Materials and Methods." Antibody staining was performed by incubation of fixed cells and frozen sections with a 1:50 dilution of a rabbit anti-rat tail tendon collagen type I antibody and a second fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Panel A: Intracellular collagen type I in untreated cardiac fibroblasts. Panel B: Decreased collagen type I staining after L-thyroxin treatment of cardiac fibroblasts. Panels C and D: Staining of collagen type I in the frozen sections of control untreated and L-thyroxin-treated hearts, respectively. All photographs are representatives of three individual experiments on triplicates of cell cultures and animals.



**FIGURE 8.**  $[^3\text{H}]$ Thymidine incorporation into the cell nuclei of thyroxin-treated and control untreated cardiac fibroblasts. Confluent quiescent cardiac fibroblasts were treated with 10 nM L-thyroxin for 22 hours. Methyl  $[^3\text{H}]$ thymidine incorporation was measured per microgram DNA in the cell lysate as described in "Materials and Methods." Data are expressed as mean  $\pm$  SEM ( $n=6$ ). \* $p<0.001$ .

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#### References

- Izumo S, Mahdavi V: Thyroid hormone receptor  $\alpha$  isoforms generated by alternative splicing differentially activate myosin heavy chain gene transcription. *Nature* 1988;334:539-542
- Tsika RW, Bahl JJ, Leinwand LA, Morkin E: Thyroid hormone regulates expression of a transfected human  $\alpha$ -myosin-heavy chain fusion gene in fetal rat heart cells. *Proc Natl Acad Sci U S A* 1990;87:379-383
- Malt RA, Speakman PT: Cellular synthesis of collagen: Ribosomal aggregates in hyperthyroidism, hypothyroidism, and ascorbic acid depletion. *Surgery* 1965;58:248-257
- Chassin JL, McDougall HA, MacKay M, Localio SA: Effect of stress upon the healing of wounds in rats. *Proc Soc Exp Biol Med* 1953;83:798
- Moltke E: Uptake of  $^{35}\text{S}$ -sulfate by healing wounds: Effect of thyroxine and ascorbic acid deficiency. *Acta Endocrinol* 1957;25: 179
- Eghbali M, Czaja MJ, Zeydel M, Weiner FR, Zern MA, Seifter S, Blumenfeld OO: Collagen mRNAs in isolated adult heart cells. *J Mol Cell Cardiol* 1988;20:267-276
- Eghbali ME, Eghbali M, Robinson TF, Seifter S, Blumenfeld OO: Collagen accumulation in heart ventricles as a function of growth and aging. *Cardiovasc Res* 1989;23:723-729
- Chapman D, Weber KT, Eghbali M: Regulation of fibrillar collagen types I and III and basement membrane type IV collagen gene expression in pressure overloaded rat myocardium. *Circ Res* 1990; 67:787-794
- Bhambi B, Eghbali M: Effect of norepinephrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am J Pathol* 1991;139:1131-1142

10. Eghbali M, Tomek R, Woods C, Bhambi B: Cardiac fibroblasts are predisposed to convert into myocyte phenotype: Specific effect of transforming growth factor-beta. *Proc Natl Acad Sci U S A* 1991; 88:795-799
11. Penttinen RP, Kobayashi S, Bornstein P: Transforming growth factor  $\beta$  increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. *Proc Natl Acad Sci U S A* 1988;85:1105-1108
12. Lichtler A, Stover ML, Angilly J, Kream B, Rowe DW: Isolation and characterization of the rat  $\alpha_1(I)$  collagen promoter. *J Biol Chem* 1989;264:3072-3077
13. Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* 1982;2:1044-1051
14. Chirgwin JM, Prazybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979;18:5294-5299
15. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159
16. Feinberg AP, Vogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983;132:6-13
17. Genovese C, Rowe D, Kream B: Construction of DNA sequences complementary to rat  $\alpha_1$  and  $\alpha_2$  collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-Dihydroxy-vitamin D. *Biochemistry* 1984;23:6210-6216
18. Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, Kirschner MW: Number and evolutionary conservation of  $\alpha$  and  $\beta$ -tubulin and cytoplasmic  $\beta$ - and  $\gamma$ -actin genes using specific cloned cDNA probes. *Cell* 1980;20:95-105
19. Derynck R, Rhee L: Sequence of the porcine transforming growth factor  $\beta$ . *Nucleic Acids Res* 1987;15:3187
20. Curran T: FOS, in Reddy EP, Skalka AM, Curran T (eds): *The Oncogene Handbook*. Amsterdam, Elsevier, 1988, pp 307-325
21. Sukhatme VP, Sreedharan K, Toback FG, Taub R, Hoover R, Tsai-Morris CH: A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. *Oncogene Res* 1987;1:343-355
22. Armitage P: *Statistical Methods in Medical Research*. Oxford, UK, Blackwell Scientific, 1971
23. Markham BE, Bahl JJ, Gustafson TA, Morkin E: Interaction of a protein factor within a thyroid hormone-sensitive region of rat  $\alpha$ -myosin heavy chain gene. *J Cell Biol* 1987;262:12856-12862
24. Eghbali M, Tomek R, Sukhatme VP, Woods C, Bhambi B: Differential effects of transforming growth factor- $\beta_1$  and phorbol myristate acetate on cardiac fibroblasts. *Circ Res* 1991;69:483-490