Regulation of Na,K-ATPase Gene Expression by Thyroid Hormone in Rat Cardiocytes

Tsuyoshi Kamitani, Uichi Ikeda, Shigeaki Muto, Kiyoshi Kawakami, Kei Nagano, Yoshio Tsuruya, Asahiko Oguchi, Keiji Yamamoto, Yukichi Hara, Toshiyuki Kojima, Russell M. Medford, and Kazuyuki Shimada

Synthesis and activity of the enzymatic equivalent of the sodium pump, Na,K-ATPase, are regulated by thyroid hormone in responsive tissues. The purpose of this study was to determine whether triiodothyronine ($T_3$) regulates the level of the messenger RNA (mRNA) coding for Na,K-ATPase $\alpha$- and $\beta$-subunits in the heart. The expression of Na,K-ATPase mRNAs in in vitro myocardial cells was directly assayed by Northern and slot blot hybridization using Na,K-ATPase $\alpha$- and $\beta$-isoform–specific cDNA probes. Exposure of cultured neonatal rat cardiocytes to $10^{-6}$ M $T_3$ resulted in 1) threefold to fourfold increase in $\alpha_1$- and $\beta_1$-mRNA accumulation, with a maximum elevation at 48 hours, 2) sevenfold increase in $\alpha_2$-mRNA accumulation with a peak elevation at 72 hours, and 3) transient threefold increase in $\alpha_3$-mRNA within the first 24 hours followed by a deinduction thereafter. The increase in $\alpha_2$-mRNA accumulation by $T_3$ occurred over the physiological $T_3$ concentration range with an $EC_{50}$ of $5 \times 10^{-10}$ M. This was associated with a twofold increase in $\alpha_2$-subunit protein accumulation and an increase in Na,K-ATPase transport activity. The half-life of $\alpha_2$-mRNA analyzed by actinomycin D chase was less than 3 hours and was not affected by $T_3$. Transfection experiments with the luciferase reporter gene revealed that thyroid hormone response sequences are located within the 5'-flanking regions of each $\alpha$-isoform gene. The above results suggest that thyroid hormone regulates all three Na,K-ATPase $\alpha$-isoforms in cardiocytes and may play an important role in the developmental switching of the cardiac $\alpha_2$- and $\alpha_3$-isoforms. These effects are mediated, at least in part, by transcriptional regulatory factors interacting with the respective $\alpha$-isoform gene promoters. (Circulation Research 1992;71:1457-1464)

**KEY WORDS** • Na,K-ATPase • sodium pump • thyroid hormone • cardiocyte • luciferase gene

Alterations in the regulation of intracellular Na$^+$ ([Na$^+$]) homeostasis are likely to be important in the control of cardiac contractility, excitability, and cell volume regulation. Na,K-ATPase, also referred to as the sarcolemmal sodium pump, plays an important role in the regulation of [Na$^+$]. The Na,K-ATPase protein comprises two subunits in a 1:1 molar ratio: a large catalytic $\alpha$-subunit ($M_\text{r}$, 112,000) and a smaller glycosylated $\beta$-subunit ($M_\text{r}$, 35,000) of unknown function. The $\alpha$-subunit contains an intracellular ATP binding site and phosphorylation site and an extracellular binding site for cardiac glycocides. At least three $\alpha$-subunit isoforms, $\alpha_1$, $\alpha_2$, and $\alpha_3$, have been characterized in rats,2,3 chickens,4 and humans.5 Recently, two types of $\beta$-isoforms ($\beta_1$ and $\beta_2$) have also been identified in rats.6 Changes in thyroid state have been well documented to affect the sensitivity of the heart to cardiac glycocides. At the cellular level, thyroid hormone has been shown to increase Na,K-ATPase activity and the number of Na,K-ATPase sites in several types of tissue, including heart cells.7-14 The exact mechanism of the induction process by thyroid hormone is not known, although the previous study of Lo and Lo15 indicates that the increase in sodium pump site density in renal cortex is a result of an increase in synthesis rate rather than a change in degradation of pump sites. Recently, Chaudhury et al16 and McDonough et al17 have reported that thyroid hormone stimulates Na,K-ATPase gene expression in in vivo rat heart and kidney; however, it is not known whether thyroid hormone controls Na,K-ATPase gene expression directly or whether it has a posttranscriptional effect in those tissues.

In the studies reported here, we have investigated regulatory mechanisms of Na,K-ATPase gene expression by thyroid hormone in rat cardiocytes in vitro. We show that thyroid hormone directly controls Na,K-ATPase gene expression at a transcriptional level, which in turn alters intracellular ion compositions and physiological functions of cardiocytes.

From the Departments of Cardiology (T. Kamitani, U.I., Y.T., A.O., K.Y., K.S.), Nephrology (S.M.), and Biology (K.K., K.N.), Jichi Medical School, Tochigi, Japan; the Department of Biochemistry (Y.H., T. Kojima), Tokyo Medical and Dental University School of Medicine; and the Department of Medicine (R.M.M.), Emory University School of Medicine, Atlanta, Ga.

Supported by the Ministry of Education, Culture, and Science (grants 02670407 and 04770551), the Yamanouchi Foundation for Research on Metabolic Disorders, the Kanae New Drug Foundation, and the Japan Heart Foundation.

Address for correspondence: Uichi Ikeda, MD, PhD, Department of Cardiology, Jichi Medical School, Minamakawachi-Machi, Tochigi 329-04, Japan.

Received May 15, 1991; accepted September 4, 1992.
Materials and Methods

Culture of Neonatal Rat Cardiocytes

Primary cardiocytes from 1-day-old Sprague-Dawley rats were prepared by the method of Bloch et al.18 with minor modification. Briefly, cell suspensions after 0.4% trypan blue exclusion were washed with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7% fetal calf serum (FCS), plated on 100-mm culture dishes, and incubated for 2 hours at 37°C. During this interval, mesenchymal cells and some few myocytes attached to culture dishes, but most myocytes remained suspended in the medium. The medium containing myocytes was pooled and transferred to 60-mm dishes at a density of 4×10^6 cells per dish in 7% FCS containing DMEM supplemented with thymidine (0.6 mg/ml), penicillin (20 IU/ml), streptomycin (20 μg/ml), and gentamicin (20 μg/ml). After 48 hours’ incubation, the medium was changed to serum-free DMEM supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), and the above-mentioned antibiotics. Cells were used for experiments after 24-hour incubation in serum-free DMEM.

Fluorescent Flow Cytometry

Cells cultured on dishes were fixed in 2 ml of 1% paraformaldehyde in phosphate-buffered saline (PBS) and 0.1% Triton X-100 for 10 minutes at 4°C. After one washing with PBS, cells were incubated in 2 ml of PBS containing 1% bovine serum albumin and either a Na,K-ATPase α₁-subunit monoclonal antibody (9A-5, mouse IgG1)19 or a pan-myosin heavy chain (MHC) monoclonal antibody (mouse IgG1, Amersham). After incubation for 1 hour at room temperature, cells were washed twice with PBS and then incubated in 2 ml of biotinylated anti-mouse IgG1 (Amersham, 1:100 in PBS) for 1 hour at room temperature, followed by addition of 2 ml of fluorescein-streptavidin (Amersham, 1:100 in PBS) for 1 hour at room temperature. After two washings with PBS, cells were detached from the plates with 0.125% trypsin solution. The fluorescein-streptavidin-labeled cells (1×10^6) were analyzed with a flow cytometer (FACScan, Becton Dickinson) by logarithmic integral green fluorescence after gating on forward-angle light scatter. Because all patterns approximated normal curves, the peak fluorescence was taken as the fluorescent channel with the highest number of cells. Peak fluorescence was then converted from channel units of logarithmic fluorescence to linear units and used to derive percentage of maximal peak fluorescence.

Northern Blot Analysis

RNA was prepared by the guanidine isothiocyanate–cesium chloride (GITC-CsCl) method. Equal amounts of total RNA (10–15 μg) were size fractionated by electrophoresis on denaturing 1.0% agarose/formaldehyde gels and transferred to nylon membranes (Hybond N⁺, Amersham). Hybridizations were performed at 65°C for 24 hours with an excess of 32P-deoxycytidine-5’-triphosphate (dCTP)–labeled rat Na,K-ATPase α₁- and β₁-subunit isofrom complementary DNA (cDNA) probes (specific activity >1×10^6 cpm/μg DNA) by use of a multiprimer DNA labeling kit (Amersham). The α₁-cDNA probe consisted of a 2.2-kb Nco I–Bgl II restriction fragment.20 The α₁-cDNA probe consisted of a 2.9-kb fragment restricted by Sac I.2 The α₁-cDNA consisted of a 1.6-kb Sal I–EcoRI restriction fragment.2,21 The β₁-cDNA probe consisted of a 0.9-kb Pst I restriction fragment.22 The filters were washed twice in 0.2×SSC at 65°C (1×SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Autoradiography was performed at −70°C overnight and quantified by densitometric scanning (Bromma 2202 Ultrosan).

Slot blot Analysis

RNA samples were denatured by heating at 65°C for 5 minutes and cooled on ice. Aliquots containing 5 μg total RNA were spotted directly onto nylon membranes. After washing with 20×SSC, filters were hybridized to 32P-dCTP-labeled Na,K-ATPase cDNA probes. Washing and autoradiography were performed as described in “Northern Blot Analysis.”

Sodium Content

The cell monolayers were washed three times (within 15 seconds) with ice-cold 100 mM MgCl₂. The washed cells were air-dried and extracted overnight in 1.5 ml of 3N HNO₃. Extract (500 μl) was diluted to 1 ml with Na⁺- and K⁺-free solution (0.02% Acetoinox) and analyzed for intracellular sodium content ([Na⁺]) with an atomic absorption spectrophotometer (model 460, Perkin-Elmer).23 Na⁺ in the standard solution used for calibration ranged from 20 to 120 mM. After the residual HNO₃ solution was removed from the culture plates, 2 ml of 0.2N NaOH was added to dissolve the cells, and protein content was assayed by the method of Lowry et al.24 The sodium content was calculated and expressed as nanomoles per milligram protein.

Constitutes

HindIII fragments (1.8-kb) of rat Na₃K-ATPase α₁-isofrom,25 2.5-kb BstEII fragments of α₁-isofrom,26 and 2.6-kb HindIII–Sac II fragments of α₁-isofrom27 5’-flanking regions containing the translation initiation sites were subcloned into the 5’ end of the luciferase coding sequence in the plasmid pSV0A/LΔS’. The pSV0A/LΔS’ construct contained the entire coding sequence of the luciferase gene minus its promoter.28

Transfection Experiments

For transfection experiments, we used six constructs: pSV0A/LΔS’, the above-mentioned constructs designated PA1LF for α₁-isofrom, PA2LF for α₁-isofrom, PA3LF for α₁-isofrom genes, pSV2A/LΔS’, and pSV2CAT, which have the simian virus 40 (SV40) enhancer and early promoter sequences driving the luciferase and chloramphenicol acetyltransferase (CAT) genes, respectively. pSV2A/LΔS’ was used as the positive control, and pSV2CAT was used as an internal control of the luciferase gene expression.29 Each 6 μg of hybrid DNA per dish was cotransfected with 2 μg of pSV2CAT into cardiomyocytes by the CaPO₄ precipitation method. The medium was changed to serum-free medium after 24-hour incubation, followed by addition of T3 (10 nM) for 48 hours for the pSV2A/LΔS’, PA1LF, and PA3LF systems and for 72 hours for the PA2LF system. Cardiomyocytes were harvested and lysed, and luciferase activity was measured as described by DeWet et al.30 by use of a luminometer (model 1251, LKB Instruments) and normalized with CAT activity in the
same cell lysate.\textsuperscript{29} CAT activity was assayed as described by Gorman et al.\textsuperscript{30} The acetylated chloramphenicol was separated by thin-layer chromatography, and the radioactivity was assayed with a radioanalytic imaging system (AMBIS system, San Diego, Calif.).

Miscellaneous

Statistical analysis was performed with Student’s $t$ test. Values of $p<0.05$ were considered to indicate a statistically significant difference. Chemicals were of the highest grade commercially available. \textsuperscript{32}P-dCTP was from New England Nuclear, Boston.

Results

To study the effects of thyroid hormone on cardiocyte gene expression and function, an in vitro primary neonatal rat cardiocyte tissue culture model was developed and characterized. Because of its potential effects on gene expression and myogenesis,\textsuperscript{31} and in contrast to other cultured neonatal rat cardiocyte systems,\textsuperscript{32} we determined whether a homogeneous cardiocyte cell population could be achieved in the absence of bromodeoxyuridine (BrdU), a thymidine analogue often used to inhibit noncardiocyte cell proliferation. After transfer into serum-free defined media, the cardiocyte cell population was characterized regarding cell function and population homogeneity. One day after transfer to serum-free DMEM (defined as day 0), microscopic inspection of many fields revealed close to 100% spontaneously beating cardiocytes. This high fraction of functional myocardial cells was observed for at least 96 hours.

To determine the proportion of cardiocytes and non-cardiocytes in the cell population, MHC antibody-treated cells were analyzed by fluorescent flow cytometry at 96 hours after primary culture (Figure 1). Simultaneous analysis of myosin-associated fluorescence (x axis) and cell size (y axis) demonstrated that more than 95% of the cells expressed large amounts of MHC (Figure 1, quadrant 2); non-MHC-containing cells represented less than 5% of the total cell population (Figure 1, quadrant 1). Cellular fragments and debris constitute quadrants 3 and 4. These studies suggest that this in vitro population is highly homogeneous for functional cardiocytes with a minimal noncardiocyte cell component even in the absence of BrdU. In addition, the cardiocyte population can be maintained for extended periods in defined media.

As shown in Figure 2, Northern blots washed under stringent conditions revealed a single band for $\alpha_1$- and $\alpha_2$-messenger RNA (mRNA) and two bands for $\alpha_1$-mRNA in total RNA isolated from in vivo neonatal rat hearts and in vitro cultured neonatal rat cardiocytes. Incubation of the cultures for 96 hours in serum-free DMEM without T$_3$ caused no marked changes in $\alpha_1$-, $\alpha_2$-, and $\alpha_3$-mRNA levels.

After 24-hour preincubation in serum-free DMEM, cultured cardiocytes were exposed to T$_3$ for 72 hours (Figure 3). The stimulatory effect of T$_3$ on Na,K-ATPase $\alpha_1$- and $\alpha_2$-mRNA accumulation was not seen at 6 hours, became maximum at 48 hours in $\alpha_2$-mRNA with a fourfold increase, and became maximum at 72 hours in $\alpha_2$-mRNA with a sevenfold increase. T$_3$ caused a transient threefold increase in $\alpha_3$-mRNA levels within the first 24 hours, followed by a significant decrease thereafter. $\beta_1$-mRNA showed a similar time course to that of $\alpha_2$-mRNA with a maximum threefold increase at 48 hours (Figure 4).

In rat hearts, $\alpha_1$-mRNA is the major $\alpha$-isoform transcript through all developmental stages.\textsuperscript{33} We further investigated Na,K-ATPase $\alpha_1$-mRNA expression. The concentration–effect relation for the effect of T$_3$ on $\alpha_1$-mRNA accumulation is shown in Figure 5. In these experiments, cultured myocytes were exposed to concentrations of T$_3$ ranging from $10^{-11}$ M to $10^{-7}$ M for 48 hours. The lowest added concentration of T$_3$ that produced a maximal stimulation of $\alpha_1$-mRNA accumulation was $10^{-9}$ M; EC$_{50}$ occurred at a concentration of $5\times10^{-10}$ M T$_3$.

To determine whether thyroid hormone increased $\alpha_1$-mRNA accumulation by increasing the rate of synthesis or by decreasing the rate of degradation, cells were exposed to zero or $10^{-8}$ M T$_3$ for 48 hours and then incubated further with a high concentration of actinomycin D (5 \mu g/ml) to shut off all transcriptional activity. After 2 hours’ preincubation with actinomycin D, RNA extraction was started at time zero, and survival of $\alpha_1$-mRNA from cells taken at 2 and 4 hours during actinomycin D chase was determined on slot blots. As shown in Figure 6, $\alpha_1$-mRNA had the same short half-life ($t_{1/2}<3$ hours) in the nonexposed and T$_3$-exposed states.

To begin a study of the functional consequences of T$_3$-mediated induction of Na,K-ATPase gene expression, the accumulation of cellular $\alpha_1$-subunit was directly measured. The total amount of cellular $\alpha_1$-subunit...
increase in Na,K-ATPase ion-transport function, myocardial cells were incubated with $10^{-8}$ M T$_3$ for 48 hours, and total cellular sodium content was measured by atomic absorption spectrophotometry (Figure 8). [Na$^+$], in cells grown in serum-free, T$_3$-free medium for 0, 6, and 48 hours was 148±8.2, 150±6.0, and 154±6.8 nmol/mg protein (mean±SEM, $n=8$), respectively, indicating that growth in serum-free medium alone had no significant effect on [Na$^+$] over this period. However, the mean [Na$^+$] value in cells grown for 48 hours in $10^{-8}$ M T$_3$-containing medium was 132±3.9 nmol/mg protein (mean±SEM, $n=8$), a 15% difference ($p<0.05$) compared with 48-hour control samples. These results suggest a high level of Na,K-ATPase ion-transport activity in T$_3$-exposed versus control cells and also suggest that T$_3$-induced stimulation of Na,K-ATPase mRNA expression was not preceded by changes in [Na$^+$].

The role of thyroid hormone in the control of $\alpha$-isoform gene expression was evaluated by transfection of chimeric plasmids containing 5'-flanking sequences of rat $\alpha_1$, $\alpha_2$, and $\alpha_3$-isoform genes into primary cultures of neonatal rat cardiocytes. The genomic DNA fragments, containing 5'-flanking sequences of each $\alpha$-isoform gene, were ligated into pSV0A/LA5', a eukaryotic expression vector in which the expression of luciferase serves as a marker of gene activation. When 10 nM T$_3$ was added to the system, an approximately twofold induction of luciferase activity was observed in the $\alpha_1$-isoform system, ninefold induction was observed in the $\alpha_2$-isoform system, and threefold induction was observed in the $\alpha_3$-isoform system, whereas the luciferase activity was not affected by T$_3$ in the pSV2A/LA5' system (Figure 9). These results indicate that the DNA sequences required for induction by T$_3$ are contained within the 5'-flanking sequences of each $\alpha$-isoform gene.

**Discussion**

Sensitivity of the heart to digitalis glycosides in vivo depends in part on the thyroid state, because hyperthyroidism is associated with increased tolerance to cardiac glycosides and hypothyroidism with decreased tolerance. The results of the present study show that thyroid hormone directly regulates Na,K-ATPase $\alpha$- and $\beta$-subunit gene expression in cardiocytes, which in turn alters intracellular ion composition. It should be emphasized that this is by no means the only alteration in cardiac myocyte gene expression brought about by exposure to thyroid hormone. Changes in contractile protein isoform expression as well as sarcoplasmic reticulum Ca-ATPase, $\beta$-adrenergic receptors, and dihydropyridine-sensitive calcium channel$^{38}$ have been described in isolated cardiac cell preparations.

In this study, primary culture of neonatal rat cardiomyocytes routinely yielded preparations in which more than 95% of the cells were cardiomyocytes, as judged by interaction with a monoclonal antimitosin antibody; thus, it is unlikely that the minor degree of fibroblast contamination accounts for the T$_3$ response. Our approach also rules out indirect effects of thyroid hormone mediated by changes in hemodynamics or other mechanisms. The stimulatory effect of T$_3$ on $\alpha_1$, $\alpha_2$, and $\beta$-mRNA accumulation was not yet evident at 6 hours but became maximal by 48–72 hours. This time course was much more gradual than the response to increased.
[Na\textsuperscript{+}], induced by low [K\textsuperscript{+}], or aldosterone treatments, which were associated with a peak $\alpha_1$-mRNA accumulation within 1 or 6 hours, respectively.\textsuperscript{23,39} In contrast, $\alpha_2$-mRNA showed a rapid response to T\textsubscript{3}, with a transient peak increase within the first 24 hours followed by the deinduction thereafter.

Recently, Orlowski and Lingrel\textsuperscript{32} also showed that thyroid hormone regulates Na,K-ATPase gene expression in cultured neonatal rat cardiocytes. However, their results differ from ours in the following points: 1) Incubation of cardiocytes for 8 days in the presence of 1 $\mu$M T\textsubscript{3} caused a marked elevation in $\alpha_2$-mRNA levels of approximately sevenfold, reaching maximum expression within 2 days and sustained thereafter. 2) T\textsubscript{3} showed no effects on $\alpha_1$-mRNA levels during the 8-day incubation. We do not know the reasons for the discrepancy in the results; possible explanations are that physiological doses of T\textsubscript{3} were used in our study and that our cardiocytes were spontaneously beating during the experiments, whereas we suppose theirs were not, because cultured neonatal rat cardiocytes do not exhibit spontaneous contraction for more than several days in the absence of serum (T. Kamitani et al, unpublished observations). Hensley et al\textsuperscript{40} and Gick et al\textsuperscript{41} have reported that T\textsubscript{3} caused an increase in Na,K-ATPase $\alpha_1$, $\alpha_2$, and $\beta_1$-mRNA expressions in in vivo adult rat hearts. We have also observed that T\textsubscript{3} stimulates the $\alpha_1$-mRNA expression in adult rat ventricular cells in long-term culture.\textsuperscript{42}

Titration experiments demonstrated that this response occurred at an EC\textsubscript{50} of 0.5 nM T\textsubscript{3}. Because of possible metabolism of T\textsubscript{3} by cells in culture,\textsuperscript{43} the hormone concentrations may not have been constant over the 48-hour interval between T\textsubscript{3} additions. Nevertheless, the subnanomolar concentration of T\textsubscript{3} that produced half-maximal stimulation is well within the physiological range of rat plasma concentration.\textsuperscript{44}

Various approaches have been used to quantify Na,K-ATPase $\alpha$-subunit binding sites. Although $[^3H]$ouabain binding is often used, this approach is
difficult in rat cardiac tissue because of the relatively low ouabain binding affinity of the rat α1-subunit. Antibody-labeled flow cytometric analysis allows for an accurate measurement of changes in total Na,K-ATPase α1-subunit protein content per cell.23 Because the monoclonal antibody used, 9-A5,19 is specific for the α1-subunit, we have demonstrated a T3-mediated induction in α1-protein accumulation per cardiocyte. After 72 hours, a twofold increase in cellular α1-protein content was measured. It is likely that this represents a specific increase in the expression of Na,K-ATPase α1-protein in the cardiocytes.

Actinomycin D blocks transcription of DNA and the synthesis of mRNA by binding to the guanosine residues. It has been shown that actinomycin D could be used to determine mRNA turnover without grossly affecting posttranscriptional steps leading to its degradation.8 Our actinomycin D chase study demonstrated that T3 did not have an appreciable effect on the α3-mRNA turnover rate. Therefore, the observed accumulation of α3-mRNA in response to T3 resulted from an increase in mRNA synthesis at either the transcriptional or RNA processing steps, rather than a decrease in the mRNA degradation rate.

In rat hearts, fetal-type α1-isoform is expressed only at fetal and early neonatal stages, and α1-isoform is replaced by adult-type α1-isoform after birth.33 In the present study, T3 caused an induction of α1-isoform and

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Graph showing time course of induction of Na,K-ATPase α-subunit messenger RNA (mRNA) accumulation. α1- (○), α2- (●), and α3- (△) mRNA levels in cardiocytes exposed to 10^{-8} M T3 for the indicated times were determined by Northern blots and quantified by densitometric scanning. The relative increase was related to the mRNA levels of zero time samples. Each point is the mean ± SEM of three to five separate experiments.

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Graph showing concentration dependence of α1-messenger RNA (mRNA) accumulation by thyroid hormone. Total RNA (5 μg) from cells incubated for 48 hours in the presence of zero to 10^{-7} M T3 was immobilized on nylon membranes and hybridized to ^32P-labeled α1-subunit cDNA probe. The points represent the means of three samples.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Graph showing α1-messenger RNA stability in the presence or absence of thyroid hormone. Cells were exposed for 48 hours to zero (○) or 10^{-8} M (●) T3 and were incubated further with actinomycin D (5 μg/ml) for the indicated times. RNA extraction was started 2 hours after exposure to actinomycin D. For each time point, 5 μg total RNA was prepared and analyzed by slot blot. Autoradiography was quantified by densitometric scanning. Each point is the mean of two separate experiments.

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Bar graph showing immunofluorescence analysis of T3-mediated induction of α1-subunit accumulation. Cardiocytes were exposed to 10^{-8} M T3 for 72 hours. The cells were then permeabilized and incubated for 1 hour at 4°C with a 1:100 dilution of Na,K-ATPase α1-subunit-specific monoclonal antibody 9-A5. The cells were washed with phosphate-buffered saline and then exposed to a 1:100 dilution of biotinylated anti-mouse IgG1 followed by fluorescein-streptavidin (1:100) for 1 hour at room temperature. The cells were washed twice and analyzed with a fluorescence flow cytometer. Peak fluorescence was converted from channel units of logarithmic fluorescence to linear units, and the results were expressed as the percent of peak fluorescence observed in control samples (no T3). Each bar represents the mean ± SEM of four samples. *p<0.01, **p<0.001 vs. 0-hour control.
deinduction of α3-isofrm mRNA, supporting the premise that thyroid hormone regulates the "developmental switching" of Na,K-ATPase α-isofrm expression. This possibility is further suggested by the observations that thyroid hormone concentrations in the serum of neonatal rats are negligible at birth and increase dramatically during the first 2 weeks after birth. Recently, Melikian and Ismail-Beigi have reported that treatment of in vivo neonatal rats with T3 for 48 hours stimulated cardiac Na,K-ATPase α1-, α2-, and β1-mRNA expresions, compatible with our results in vivo; however, T3 did not affect α-mRNA expression in their system. We speculate that T3 could suppress α2-mRNA expression in their system if rats were treated with T3 for more than 48 hours.

Our findings that cell growth in a medium containing high concentrations of T3 for 48 hours was associated with lower [Na+]i support the view that T3 causes an induction of additional sodium pump sites that are functional. Similar results in [Na+]i have been observed in earlier studies from another laboratory and in still earlier studies of the diaphragm and heart of rats given T3 in vivo, although contrasting results have also been reported for mouse diaphragm in which T3 was reported to have no measurable effect on [Na+]i.

It has not been clear whether T3 acts directly to modulate Na,K-ATPase α-isofrm and β1-mRNA levels or through a combination of direct and indirect influences. For example, T3 stimulation of ion fluxes may indirectly lead to an ion-dependent increase in Na,K-ATPase expression. In support of this hypothesis, Everts and Clausen found, in rats treated with T3, a rise in passive leaks of Na+ and K+ from skeletal muscle that preceded increases in ouabain binding. However, Philipson and Edelman showed that T3-induced stimulation of Na,K-ATPase activity in hearts was not preceded by changes in [Na+]i, compatible with our results in vitro (Figure 8).

Examination of the 5' ends of rat Na,K-ATPase α1-, α2-, and α3-isofrm genes with the thyroid hormone receptor consensus binding sequence 5'-GGG(A,T)C(G,C)-3' and its reverse complement have shown that α2-26 and α3-27 isofrm genes have the sequences identical to this element, although the presence of this sequence within the 5'-flanking region of α3-isofrm gene is still controversi- al.25,52 Our transfection study supports the premise that thyroid hormone response sequences are located within the 5'-flanking sequences of each α-isofrm gene.

In conclusion, thyroid hormone regulates all three Na,K-ATPase α-isofrms in cardiocytes and may play an important role in the developmental switching of the cardiac α2- and α3-isofrms. These effects are mediated, at least in part, by transcriptional regulatory factors interacting with the respective α-isofrm gene promoters.

Acknowledgments
We thank Mary O'Neill and Toshiko Kanbe for their technical assistance and Dr. H.L. Leffert for the 9-A5 antibody.

References


Regulation of Na,K-ATPase gene expression by thyroid hormone in rat cardiocytes.
T Kamitani, U Ikeda, S Muto, K Kawakami, K Nagano, Y Tsuruya, A Oguchi, K Yamamoto, Y Hara and T Kojima

doi: 10.1161/01.RES.71.6.1457

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/71/6/1457

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/