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Thyroid Hormone Receptor α_1 Regulates Expression of the Na^+/H^+ Exchanger (NHE1)*

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In this paper we examine the role of thyroid hormone in regulating expression of the Na^+/H^+ exchanger. Thyroid hormone has been reported to regulate the activity of the Na^+/H^+ exchanger messenger RNA in some cell types. Treatment of cardiac myocytes with 3,5′,3′-triiodothyronine results in an increased expression of Na^+/H^+ exchanger protein. Also, compared with euthyroid animals, hypothyroid rats express decreased amounts of the Na^+/H^+ exchanger protein. To examine the mechanisms involved in regulating expression of the Na^+/H^+ exchanger, we have characterized the regulation of a distal element of the NHE1 promoter by the thyroid hormone receptor. We have previously shown that a −1085/−800 nucleotide (nt) region of the promoter is a modular element with a −841/−800 nt activating element. Using electrophoretic mobility shift assay, we show that this element interacts with thyroid hormone receptor TRα_1, a nuclear hormone receptor. The addition of exogenous TRα increased transcriptional activity of the −841/−800 nt element of the Na^+/H^+ exchanger promoter. We show that TRα binds to a region on the −841/−800 nt element that is near, but not identical, to the previously identified chicken ovalbumin upstream promoter transcription factor-binding site. Our results are the first demonstration that thyroid hormone and the thyroid hormone receptor TRα_1 regulate expression of the Na^+/H^+ exchanger.

The Na^+/H^+ exchanger is a plasma membrane protein that removes an intracellular proton, exchanging it with an extracellular sodium. By doing so, the Na^+/H^+ exchanger raises intracellular pH and so, not surprisingly, it responds to intracellular acidification with increased activity. In addition, Na^+/H^+ exchange activity is stimulated by a variety of growth factors (1). There are several isoforms of the Na^+/H^+ exchanger: NHE1–NHE7. NHE1, which was the first isoform cloned (2), is ubiquitously expressed in the plasma membrane of mammalian cells, whereas the other isoforms show more restricted tissue distributions (3). The Na^+/H^+ exchanger is important in many cell types in raising intracellular pH during cell growth and differentiation (4, 5). It is also involved in the damage that occurs to the myocardium during ischemia and reperfusion. As a result of this, inhibition of the Na^+/H^+ exchanger is cardioprotective, and new inhibitors are currently being developed for clinical use (6).

The regulation of expression of the Na^+/H^+ exchanger has not yet been thoroughly studied. It is known that expression of the exchanger is elevated during cellular differentiation (5, 7). Acidosis and ischemia have also been reported to increase the expression of NHE1 in some cell types, including the kidney, the lymphocytes, and the myocardium (9–11). More recently, hypertrophy has been shown to increase expression of NHE1 (12), and the Na^+/H^+ exchanger has been implicated in the etiology of hypertrophy and in ischemic heart disease (13). We have recently demonstrated that message levels for NHE1 are increased in the hearts of hyperthyroid rats (14).

The promoter-transcription factor interactions that lead to transcriptional regulation of the NHE1 gene are only now beginning to be understood. Proximal elements involved in regulation of NHE1 expression include AP-1, AP-2, and CCAAT/enhancer-binding protein (15–17). In addition, we have found that a more distal serum-responsive element exists at −1085 to −800 nt[1] from the start site (18). Within this region we identified a novel enhancer element, at −841 to −800 nt, that binds chicken ovalbumin upstream promoter transcription factors I and II (COUP-TFI and COUP-TFII, respectively) and that regulates NHE1 expression (19). In this study, we show that thyroid hormone can increase expression of the Na^+/H^+ exchanger in the myocardium. We demonstrate that the novel enhancer element (at −841 to −800 nt), acting upstream of the proximal regulatory elements of the promoter, regulates expression of NHE1 in response to the thyroid hormone receptor. Our results suggest that thyroid hormone, acting through the thyroid hormone receptor, is an important regulator of Na^+/H^+ exchanger gene expression.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes were obtained from Roche Molecular Biochemicals, PerkinElmer Life Sciences, and Invitrogen. pGEM, pSP, and pGL plasmids were from Promega (Madison, WI). pTK 81 and pTK 40-CAT were generous gifts of Dr. R Rachubinski (Department Cell Biology, University of Alberta, Edmonton, Canada) and Dr. L. Belanger (University Laval, Quebec, Canada), respectively. [α-^{32}P]dCTP was purchased from ICN Biomedicals (Irvine, CA). All of the other chemicals were of analytical or molecular biology grade and were purchased from Fisher, Sigma, or BDH (Toronto, Canada). The

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[1] The abbreviations used are: nt, nucleotide(s); AT, antithrombin III; HD-PPRE, peroxisome proliferator-response element of rat hydratase dehydrogenase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; T3, 3,5′,3′-triiodothyronine; TBS, Tris-buffered saline; EMSA, electrophoretic mobility shift binding assay(s); TR, thyroid hormone receptor.

vectors for *in vitro* and mammalian expression of COUP-TFI and COUP-TFII and rat TRα_1 transcription factors have been described earlier (19, 20). CV1 cells were a gift of Dr. Mona Nemer (Clinical Research Institute of Montreal, Montreal, Canada).

Isolation of Ventricular Myocytes—Primary myocyte cultures were prepared from neonatal Sprague-Dawley rats as described previously (21). The hearts were removed from 5–6-day-old rats under aseptic conditions, and the ventricles were minced to small size. The tissue was digested with a series of collagenase (0.1%) treatments at 37 °C. Dissociated mixtures of cells were incubated in Corning T-75 culture flasks at 37 °C in a humidified atmosphere (5% CO_2, 95% air) for 20 min. During this time, nonmyocytes (fibroblasts, endothelial cells, and smooth muscle cells) attach, and most of the myocytes remain in suspension. Subsequently, the myocytes were removed and plated onto Primaria™ (Falcon) culture dishes. The myocytes were maintained for 4–5 days in medium containing Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum, 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium nonessential amino acids, 10% minimum essential medium vitamin, 0.1 mM bromodeoxyuridine, 100 μM L-ascorbic acid, and 30 mM HEPES, pH 7.1. To determine the effect of 3,5′,3′-triiodothyronine (T3) on Na^+/H^+ exchanger message levels, the cells were maintained for 24 h in serum-free medium containing the presence or absence of 10^{-7} M T3. A microsome preparation was made for the analysis of NHE1 protein expression. The cells from three to five 35-mm Petri dishes were washed with cold phosphate-buffered saline and recovered manually in the absence of trypsin. They were centrifuged at 5000 × *g* for 3 min. The pelleted cells were suspended in 5 ml of lysis buffer consisting of 10 mM Tris, pH 8.0, 25 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, and 2 mM EDTA and incubated on ice for 10–15 min. A protease inhibitor mixture was added (22), and the sample was homogenized with 40 strokes of a tight fitting Dounce homogenizer. A further 7.5 ml of lysis buffer with 250 mM sucrose and 2 mM 2-mercaptoethanol was added, and the sample was homogenized for a further 20 strokes. The sample was then centrifuged at 16,000 × *g* for 15 min, and the supernatant was spun at 137,000 × *g* for 75 min. The final pellet was suspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, and equal amounts of protein were assayed for NHE1 protein content by Western blot analysis.

Cell Culture, Cell Transfection, and Reporter Assays—NIH 3T3 and CV1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 μg/ml of penicillin G-streptomycin as described earlier (19). The cells were transiently transfected with purified plasmids (Qiagen, Chatworth, CA) at 50% confluence using $CaPO_4$ as described earlier (18). For transient transfections, 5 μg of reporter plasmid was used unless noted otherwise. Following transfection, the cells were incubated in medium containing 0.5 or 10% fetal bovine serum without phenol red (Sigma). Thirty-six hours after transfection, the cells were harvested, and the cell lysates were assayed for protein concentration, luciferase, and β-galactosidase activities as described earlier (19). The expression vectors for transcription factors (0.25–2.5 μg/plate) were added together with the promoter-reporter vectors (2.5 μg/plate).

Preparation of Total Protein from Tissues—Ventricular tissue of hearts was harvested from hypothyroid, euthyroid, or hyperthyroid rats. The tissues were placed in a buffer containing 1 M NaCl, 100 mM Tris, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 37.5 μM ALLN (calpain I inhibitor), and a proteinase inhibitor mixture for homogenization (22). The samples were homogenized at 4 °C for 30 s, incubated on ice for 30 s, and then homogenized again for 30 s using an Omni International 2000 electric homogenizer. To obtain crude membrane fractions (which contained the NHE1 protein within membranes), homogenates were subjected to a number of centrifugation steps. Initial centrifugation was for 10′ at 3,000 rpm. The pellet was discarded, and the supernatant was centrifuged at 10,000 rpm for 15 min. The resulting pellet was again discarded, and the supernatant was centrifuged at 100,000 × *g* for 1 h to obtain a fraction enriched in crude microsomes. The microsomal pellet was resuspended in the same buffer as described above with the addition of 1% SDS to aid in solubilization. The total protein was quantified using the Bio-Rad DC protein assay kit.

Thyroid Animal Models—Three groups of Sprague-Dawley rats (100–150 g) were used essentially as described earlier (14). The control group received no treatment, whereas two other groups were made hypothyroid by the addition of 0.025% methimazole to their drinking water for 4 weeks. After 4 weeks one of the hypothyroid groups was made hyperthyroid by intraperitoneal injection of 15 μg/100 g of body weight of T3 for 5 days. The thyroid status of each animal was determined by measuring serum T3 at the time of sacrifice (14).

NHE1 Immunoblots—An anti-NHE1 monoclonal antibody was purchased from CHEMICON International, Inc. to quantify NHE1 protein in crude microsomes. This antibody was generated in mice using an immunogen that consisted of the entire C-terminal hydrophilic domain of NHE1 coupled to a maltose-binding protein. Although porcine NHE1 was used as the immunogen to generate the antibody, NHE1 is highly conserved between mammalian species.

For NHE1 immunoblots, crude membrane fractions containing 60–100 μg of total protein were run on 10% polyacrylamide gels, followed by transfer to nitrocellulose membranes. The membranes were stained with Ponceau S to confirm that all of the lanes were loaded equally. The membranes were then incubated overnight at 4 °C in 10% milk with TBS, followed by washing three times for 5 min each in TBS at room temperature. The membranes were probed at 4 °C overnight with anti-NHE1 monoclonal antibody (Chemicon) (1:2000) in TBS. Following three washes of 5 min each with TBS, the membranes were incubated with goat anti-mouse antibody (1:5000) in TBS at room temperature for 1 h. After three 5-min washes, with TBS the Amersham Biosciences ECL reaction was used to visualize immunoreactivity. The blots were scanned and quantified using Image Gauge (Bio-Rad) software essentially as described earlier (23).

Construction of Plasmids—Construction of plasmids containing the Na^+/H^+ exchanger promoter fragments was as described earlier (19). Briefly, synthetic oligonucleotides were used to amplify regions −1085 to −800 of the NHE1 promoter or the −108 to −842 region. These fragments were subcloned into the luciferase reporter vector pXP1 upstream of the minimal NHE1 promoter, which contains the −92 to +24 region of the mouse NHE1 promoter (17, 19). For some experiments we amplified the −841/−800 nt element of the NHE1 promoter and inserted four tandem copies upstream of the wild type NHE1 minimal promoter. This multiple element was also inserted upstream of the thymidine kinase minimal promoter directing luciferase expression as described earlier (19).

Electrophoretic Mobility Shift Binding Assays—Electrophoretic mobility shift binding assays (EMSA) were essentially as described earlier (19). Briefly, wild type synthetic oligonucleotides or mutants of the −841/−800 nt NHE1 region were used after annealing and labeling with Klenow and [α-^{32}P]dCTP. The wild type sequence −841 to −800 and the mutants M1–M3 are: wt, $^{-841}$GGGTCTCCCT ACTGACCTCA GCCTGGTCTA GAACTCACTT^{-800}; M1, $^{-841}$GGGCGATATA ACTGACCTCA GCCTGGTCTA GAACTCACTT^{-800}; M2, $^{-841}$GGGTCTC CCT ACCAAAACCA GCCTGGTCTA GAACTCACTT^{-800}; and M3, $^{-841}$GGGTCTCCCT ACTGACCTCA GCAAACCCTA GAACTCACTT^{-800}.

DNA binding reactions were performed at room temperature using samples of reticulocyte lysates in binding buffer (5% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.0) in the presence of 1 μg of poly(dI-dC), 0.1 μg of carrier DNA (salmon sperm DNA), and 5 μg of bovine serum albumin. The electrophoresis and autoradiography conditions were as described (19). The nuclear extracts were prepared from isolated myocytes essentially as described earlier (19). *In vitro* transcription-translation assays of COUP-TFI, COUP-TFII, and TRα_1 were with a rabbit reticulocyte lysate system (Promega) as described earlier (19). The efficiency of the reaction was judged by SDS-PAGE of samples translated concomitantly with L-[^{35}S]methionine. Electrophoretic mobility shift assays used 1–3 μl of programmed lysate or unprogrammed lysate for controls, and 10 μg of nuclear extracts of myocytes were used. For some EMSA a fragment of the antithrombin III promoter (AT) (19, 20) or the peroxisome proliferator-responsive element of the rat hydratase dehydrogenase gene (HD-PPRE) (20) were used as controls for COUP-TF and TRα, respectively.

RESULTS

Previous experiments have suggested that messenger RNA levels for the NHE1 isoform of the Na^+/H^+ exchanger are increased in the hyperthyroid heart (compared with euthyroid and hypothyroid hearts) (14). We used two models to determine whether amounts of NHE1 protein are also elevated in the myocardium in response to thyroid hormone. In initial experiments we used primary cultures of neonatal cardiac myocytes (Fig. 1). Fig. 1*A* is a representative Western blot, whereas Fig. 1*B* summarizes the results of six distinct experiments. We found that treatment of isolated myocytes with T3 resulted in increased expression of the Na^+/H^+ exchanger protein relative

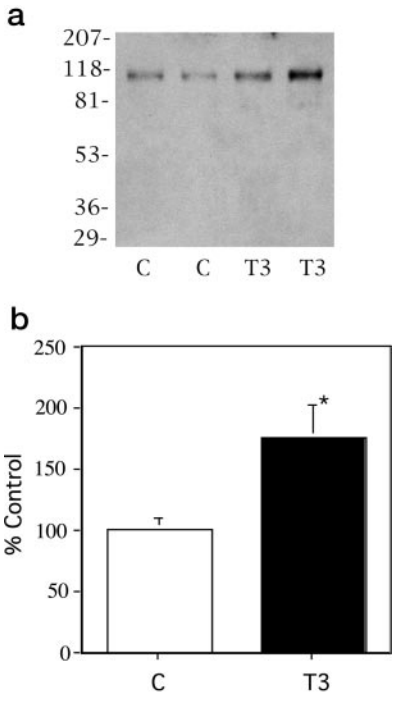

FIG. 1. **Western blot analysis of Na^+/H^+ exchanger expression in primary cultures of isolated myocytes treated with T3.** *A*, example of Western blot of control (*C*) and T3-treated (*T3*) primary cultures of isolated myocytes. *B*, summary of six experiments. *, $p < 0.05$ according to the Mann-Whitney *U* test.

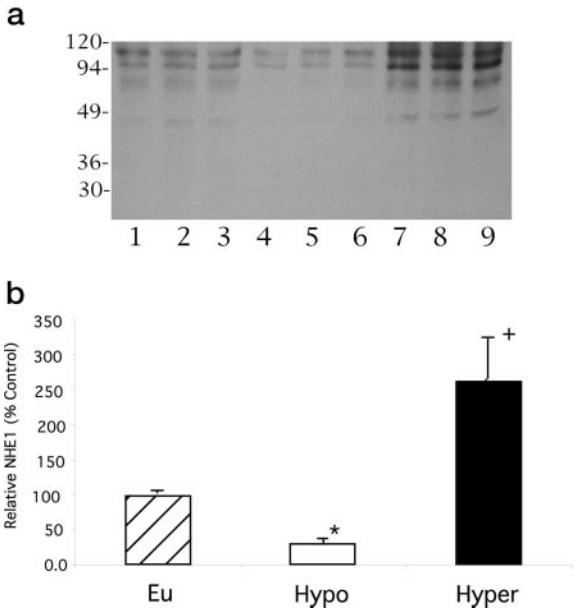

FIG. 2. **Western blot analysis of Na^+/H^+ exchanger expression in intact heart from hypothyroid, euthyroid, and hyperthyroid rats.** *A*, Western blot of microsomes prepared from euthyroid (*first* through *third lanes*), hypothyroid (*fourth* through *sixth lanes*), and hyperthyroid (*seventh* through *ninth lanes*) rat hearts. *B*, summary of experiments. +, $p < 0.05$. *, $p < 0.01$ according to the Mann-Whitney *U* test. *Eu*, euthyroid; *Hypo*, hypothyroid; *Hyper*, hyperthyroid.

to untreated cells. The treatment increased NHE1 expression by ~75%.

To confirm these results in another model, we assessed NHE1 levels in the myocardium of animals that were hypothyroid, euthyroid, or hyperthyroid. The results are shown in Fig. 2. Fig. 2*A* is a representative Western blot, whereas Fig. 2*B* summarizes the results of six distinct experiments. The hyperthyroid animals showed elevated levels of NHE1 expression compared with both euthyroid and hypothyroid animals. Further, the level of NHE1 protein was decreased in hypothyroid animals compared with the euthyroid animals. These results confirm the effects of T3 in isolated myocytes (Fig. 1) and demonstrate that expression of NHE1 in the myocardium is affected by treatment with T3. As observed typically with the Na^+/H^+ exchanger (24), we consistently observed two immunoreactive bands on our Western blots: a larger band of ~105 kDa that represents fully glycosylated NHE1 and a smaller band that represents unprocessed or only partially glycosylated protein.

In a previous experiment (19), using electrophoretic mobility shift assays, we observed that the −841/−800 element of the mouse NHE1 promoter competes with a thyroid receptor palindrome for binding proteins in NIH 3T3 nuclear extracts. This led us to suspect that the −841/−800 nt element of the NHE1 promoter might interact with the thyroid receptor. Therefore, we examined binding of TRα expressed in reticulocyte lysates to the −841/−800 nt element. We have previously shown (19) that the −841/−800 nt element binds COUP-TFI and COUP-TFII and that mutation of the element decreases this binding. Therefore, we used these proteins as controls for assessing binding of TRα to the −841/−800 nt element. The results are shown in Fig. 3. In EMSA, both COUP-TFI (Fig. 3*A*) and COUP-TFII (Fig. 3*B*) bound to the wild type −841/−800 nt element of the NHE1 promoter. As a positive control we included a human antithrombin III promoter fragment (AT), which also bound to the two isoforms of COUP-TF. We also confirmed the effect of mutating the −841/−800 nt element on these interactions. M1, M2, and M3 are three mutants of the −841/−800 nt element at positions −838/−832, −829/−824, and −819/−815, respectively. As we found previously, both isoforms of COUP-TF bound to M3, showed reduced binding to M1, and showed no binding to M2. The binding of TRα to the −841/−800 nt element of the NHE1 promoter and the effect of the three mutations on this binding are shown in Fig. 3 (*C* and *D*). In control experiments, as expected, *in vitro* translated rat TRα bound to the proximal response element HD-PPRE (Fig. 3*C*) and exhibited a supershift in the presence of anti-TRα antibodies. The *in vitro* translated TRα also bound directly to the −841/−800 nt element, and the mobility of the complex was similar to that of HD-PPRE, which binds TRα as monomer (25). TRα showed reduced binding to the M1 mutant, whereas it bound to the M2 mutant as both a monomer and a dimer, likely a homodimer. The mobility shifts resulting from TRα binding to M2 were affected by anti-TRα antibody. Anti-TRα antibody resulted in a reduction in both the dimer and monomer forms of M2 mutant (Fig. 3*E*). The M3 mutation did not affect TRα binding to the −841/−800 nt element.

In another experiment, we use EMSA to investigate TRα binding to the M3 −841/−800 nt element in competition with the M1 and M2 elements (in 100-fold excess). In the presence of M2, binding of TRα to M3 was 94% of that without competition. In contrast, in the presence of M1, binding of TRα to M3 was 53% of that without competition. These results suggest that the region of the M2 mutation is not involved in binding TRα, whereas the region of the M1 mutation is at least partially involved. These results were consistent with the results shown in Fig. 3*D*.

Fig. 3*F* illustrates the results of testing nuclear extracts of isolated myocytes with the −841/−800 nt element. *Lane 1* shows the labeled probe alone. *Lane 2* shows that nuclear extracts of myocytes treated with T3 show significant binding to the labeled −841/−800 nt element. Competition with unlabeled −841/−800 nt element eliminated the binding completely (*Lane 3*). *Lane 4* shows that nuclear extracts from

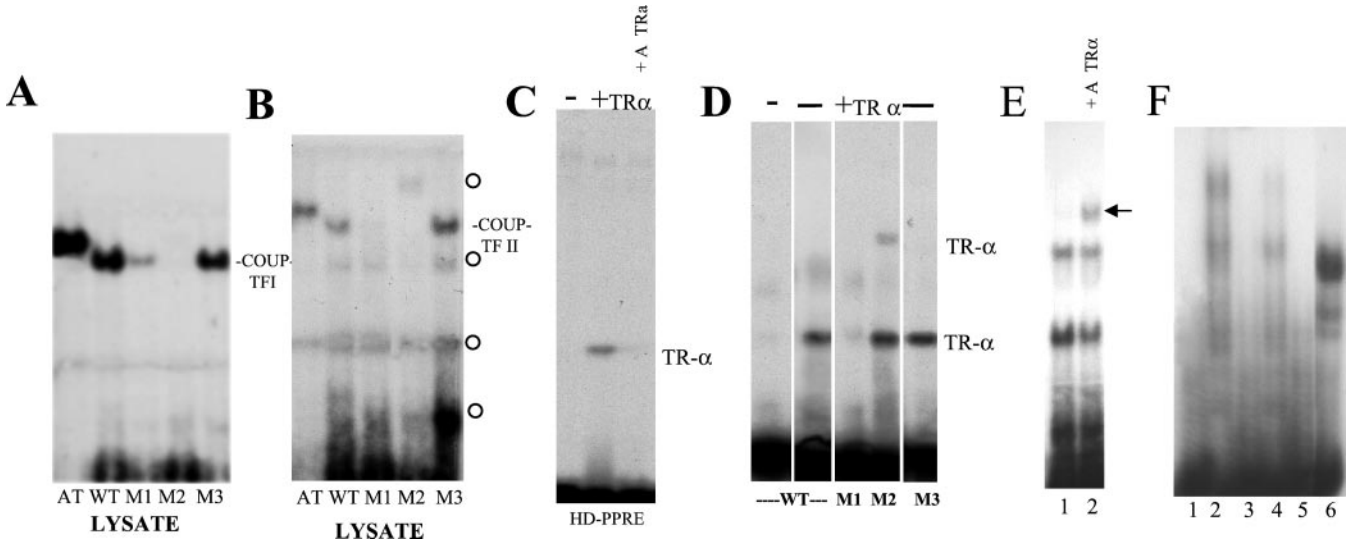

FIG. 3. ***In vitro* binding of COUP-TFs and TRα to control response elements (AT and HD-PPRE) and to wild type and mutated −841/−800 nt elements WT, M1–M3.** *A*, binding of *in vitro* translated COUP-TFI to AT, control, and mutants M1–M3. *B*, binding of *in vitro* translated COUP-TFII to AT, control, and mutants M1–M3. Binding of COUP-TFII by mutant M1 was weak and was not as clearly visible in some instances but was visible at other times. The *open circles* indicate nonspecific bands. *C*, TRα antibody reactivity with control element (HD-PPRE) in samples of reticulocyte lysate programmed (+) or unprogrammed (−) with TRα. Reactivity with TRα antibody (+*A TR*α) was indicated by a decrease in the amount shift-reactive species present. *D*, binding of *in vitro* translated TRα to wild type and mutated −841/−800 nt elements WT, M1–M3. Note the two bands present in reactions with M2, which are indicated in the margin. *E*, TRα antibody reactivity with M2 element in samples of reticulocyte lysate programmed with TRα. *Lane 1*, M2 element with programmed lysate. *Lane 2*, M2 with programmed lysate and anti-TRα antibody (+*A TR*α). The *arrow* denotes the supershifted species. *F*, *in vitro* binding of nuclear extracts from primary cultures of isolated myocytes treated with T3 (as described for Fig. 1). All of the *lanes* contain wild type −841/−800 nt element. *Lane 1*, no nuclear extract. *Lane 2*, nuclear extract of T3-treated myocytes. *Lane 3*, nuclear extract of T3-treated myocytes in the presence of 100X excess of cold competitor. *Lane 4*, nuclear extract of control myocytes. *Lane 5*, nuclear extract of control myocytes in the presence of 100× excess of cold competitor. *Lane 6*, binding of *in vitro* translated TRα. *WT*, wild type.

untreated myocytes show reduced binding relative to T3-treated myocytes, although the same pattern of binding was present. *Lane 6* shows the binding of *in vitro* translated TRα.

Next, we investigated whether COUP-TF and TRα might form heterodimers on the −841/−800 nt element. The wild type −841/−800 nt element binds both proteins independently (Fig. 4). As found earlier, M1 showed reduced binding of both COUP-TFI (reduced 60–70%) and TRα reduced 95%); M2 did not bind COUP-TFI but did bind TRα, as both a monomer (40%) and a dimer (60%); and binding of TRα and COUP-TF1 to M3 is unaltered compared with binding to the wild type element. Next, we looked at whether binding of either TRα or COUP-TFI to the −841/−800 nt element affects binding of the other transcription factor (Fig. 4*B*). COUP-TFI and TRα were prepared in reticulocyte lysates, as described above, and EMSAs were carried out with the wild type −841/−800 nt element. We found that TRα does interfere with the binding of COUP-TFI to the −841/−800 nt element. *Lanes 2* and *3* of Fig. 4*B* both clearly demonstrate reduced COUP-TFI binding compared with that in *lane 1* (no addition of TRα). The maximal reduction in COUP-TFI binding was ~60%. This declined to ~15% with the lowest dose of TRα. The inhibition of COUP-TFI binding was lessened when smaller amounts of TRα were added (*lanes 3–5*). In contrast, using a similar assay, we found that COUP-TF1 does not significantly reduce binding of TRα (Fig. 4*B*, *lanes 10–15*).

To investigate the effects of TRα on the Na^+/H^+ exchanger promoter *in vivo*, we carried out a co-transfection experiment using a luciferase reporter gene system. We co-transfected an expression vector for TRα with a vector containing the luciferase gene driven by a minimal NHE1 promoter and tandem upstream copies of the −841/−800 nt element (Fig. 5). Co-transfection with TRα increased luciferase activity generated by the −841/−800 nt element, and this effect was slightly more noticeable in 10% serum than in 0.5%. In contrast, TRα did not affect basal luciferase activity driven by the thymidine kinase promoter or by the minimal NHE1 promoter without the −841/−800 nt element (results not shown). It was noticeable that transfection with larger amounts of TRα (2.5 μg) had no effect on NIH3T3 cells and reduced effects in CV1 cells compared with transfection with smaller amount of the plasmid (1.25 μg). In the absence of exogenous TRα, and in 10% serum, the luciferase activity generated by the four tandem copies of the −841/−800 nt element upstream of the NHE1 minimal promoter was about double that obtained in 0.5% serum. Luciferase activity, with 10% serum and 1.25 μg of plasmid, was as follows: 404 ± 11 and 226 ± 24% in CV1 and NIH 3T3 cells, respectively. In 0.5% serum these values were 335 ± 18 and 186 ± 26% for CV1 and NIH 3T3 cells, respectively. The activating effect of TRα was also seen when it was co-transfected and expressed in the pRc/RSV system (Invitrogen; data not shown).

In these *in vivo* experiments, to confirm that TRα was acting through the −841/−800 nt element of the NHE1 promoter, we also looked at the effects of COUP-TF-1 and II and TRα on luciferase expression driven by the −1085/−842 and −1085/−800 nt elements upstream of the NHE1 minimal promoter (Fig. 6). A single copy of the −1085/−800 nt element enabled increased transcription from the NHE1 minimal promoter in response to both COUP-TF and TRα. In contrast, when the −1085/−842 element was included upstream of the minimal promoter, neither COUP-TF-1 nor TRα affected rates of transcription. These results indicate that the −841/−800 nt element is critical in activation of the NHE 1 promoter by COUP-TF-1 and TRα.

Finally, we looked at the combined effect of COUP-TF and TRα on transcriptional activity of the −841/−800 nt element. NIH3T3 and CV1 cells were transfected with expression vectors for COUP-TF-I, for TRα, or for both, and the effect of these transfections on the NHE1 minimal promoter and on the thymidine

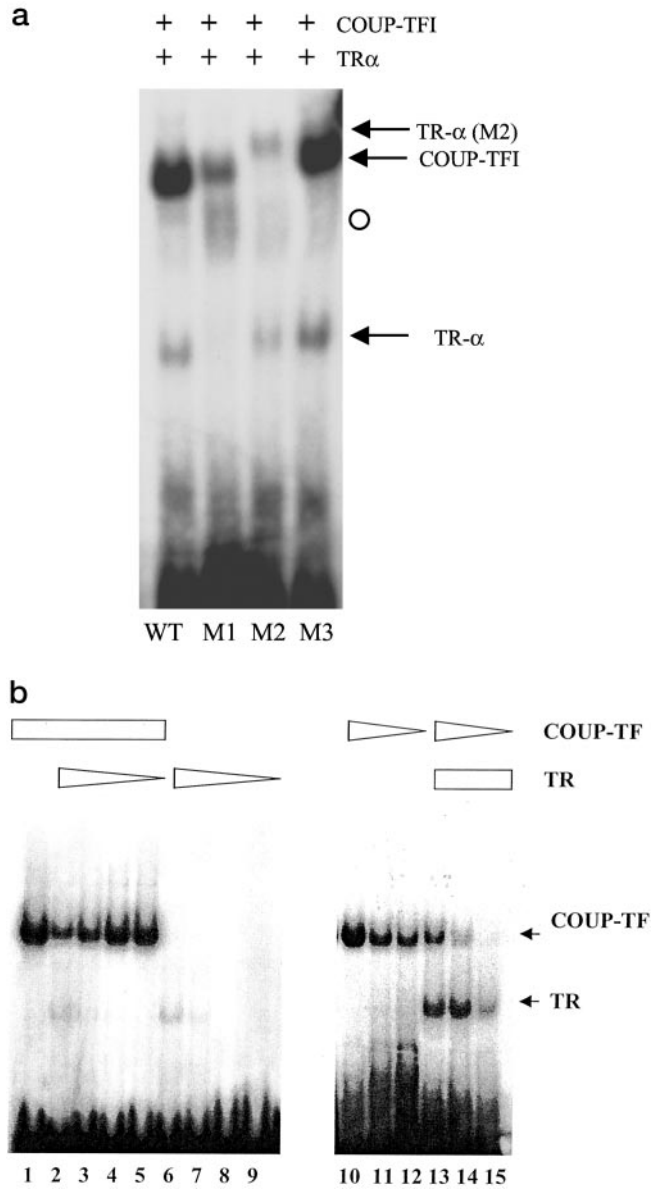

FIG. 4. **Examination of potential heterodimerization and competition between COUP-TFI and TRα.** *A*, *in vitro* binding of COUP-TFI and TRα expressed in reticulocyte lysates to wild type and mutated −841/−800 nt elements. WT, wild type. M1, M2, and M3, mutants M1–M3 of the −841/−800 nt element. The *open circle* indicates nonspecific band. The *arrow* labeled *TRα(M2)* indicates a dimer of the TRα protein on *lane M2*. *B*, COUP-TFII and TRα were prepared in reticulocyte lysates, and electrophoretic mobility shift assays were used with the −841/−800 nt element as described above. *Lanes 1–5* contained a constant amount of COUP-TF (1 μg of lysate). *Lane 1* contained no TRα, and *lanes 2–5* contained decreasing amounts of TRα (2.5, 1, 0.5, and 0.25 μg of lysate, respectively). *Lanes 6–9* contained decreasing amounts of TRα in the absence of COUP-TFII (2.5, 1, 0.5, and 0.25 μg of lysate, respectively). *Lanes 10–12* contained decreasing amounts of COUP-TFII in the absence of TRα (1, 0.6, and 0.3 μg of lysate, respectively). *Lanes 13–15* contained decreasing amounts of COUP-TFII (1, 0.6, and 0.3 μg of lysate, respectively) and a constant amount of TRα (1.5 μg of lysate).

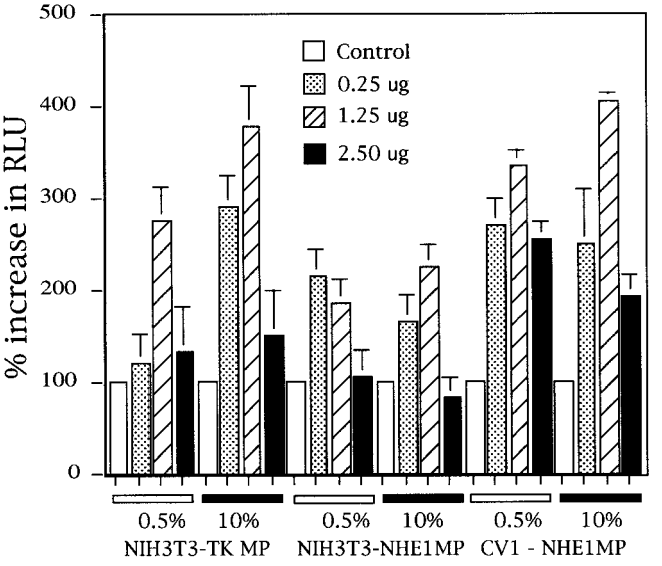

FIG. 5. **Effect of TRα on reporter activity of the −841/−800 nt element in the presence of 0.5 or 10% fetal bovine serum.** The expression vector for the TRα receptor was co-transfected in CV1 or NIH3T3 cells with the NHE1 reporter plasmid that included four tandem copies of the −841/−800 nt wild type element upstream of the NHE1 minimal promoter or a truncated version of the thymidine kinase promoter. Each plate was transfected with 2.5 μg of reporter plasmid in the presence of 0.25, 1.25, or 2.5 μg of expression vector for the TRα nuclear receptors. In all cases a constant amount of expression vector was maintained by co-transfection of the empty expression vector pSG5. The base-line values used for normalization in the absence of TRα were measured in the presence of 2.5 μg of pSG5. The activity indicated as 100% in the Fig. is the relative light unit (*RLU*) value given by four tandem copies of the −841/−800 nt element upstream of either the NHE1 minimal promoter. The results are the means ± S.E. of at least four determinations.

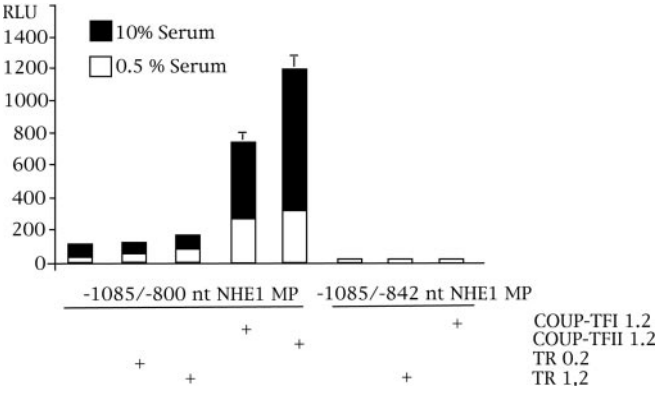

FIG. 6. **Effects of COUP-TFI, COUP-TFII, and TRα on luciferase activity influenced by the −1085/−800 nt and the −1085/−842 nt elements with the minimal NHE1 promoter.** NIH 3T3 cells were transfected with 2.5 μg/plate of reporter plasmid carrying single copies of the −1085/−800 nt or the −1085/−842 nt elements of the NHE1 promoter in front of the NHE1 minimal promoter (−92 to +24 nt). In addition 0.2–1.2 μg of expression vector for the COUP-TF or TRα were co-transfected. A constant amount of expression vector was maintained by co-transfected the empty pSG5 vector. The base-line values were in the presence of pSG5 alone.

kinase minimal promoter was measured. Table I summarizes our results. In both cell types, transfection with COUP-TFI and TRα together resulted in a greater increase in promoter activity than seen with either element alone. That is, co-expression of the two hormone receptors resulted in a slight synergism.

DISCUSSION

The regulation of expression of the Na^+/H^+ exchanger (NHE1 isoform) is of great importance for a variety of reasons. The Na^+/H^+ exchanger is involved in the growth and development of a variety of cells, and in the myocardium it has been implicated in both hypertrophy and ischemic reperfusion damage (1, 6, 12). A number of preliminary observations led us to investigate the role of thyroid hormone in expression of the Na^+/H^+ exchanger. First, thyroid hormone is known to have diverse effects on the myocardium and other tissues. T3 (the most active form of the hormone) regulates many aspects of cellular development and homeostasis. For example, in the

TABLE I

Effect of cotransfection of TRα and COUP-TF on enhancer activity of the NHE1 −841/−800 nt element in NIH3T3 and CV1 cells

The cells were transfected with either TRα (0.2 μg) or COUP-TFI (1.25 μg) expression plasmids as described for Fig. 5. The plasmids used for expressing luciferase activity contained four copies of the −841/−800 nucleotide element in front of either the minimal NHE1 promoter or the minimal thymidine kinase promoter as described for Fig. 5. A luciferase activity of 100% was assigned to the values obtained when transfecting with the plasmid containing four copies of the −841/−800 nucleotide element plus the minimal NHE1 promoter (−92/+24 NHE1-pXP1luc).

Cell line	Promoter	Luciferase activity		
		COUP-TFI (1.25 μg)	COUP-TFI (1.25 μg) and TRα (0.2 μg)	TRα (0.2 μg)
CV1	NHE1	362	837	153
NIH3T3	NHE1	281	485	142
NIH3T3	TK	844	1957	124

myocardium T3 is known to cause shifts in the type of myosin heavy chain that is expressed (26) and in expression of the Ca^{2+}-ATPase (27). Second, several earlier studies have suggested that thyroid hormone affects levels of expression of the Na^+/H^+ exchanger and its function. For example, in L-6 cells, T3 and L-thyroxine (T4) directly stimulate activity of the Na^+/H^+ exchanger (28). Thyroid hormone has also been shown to increase Na^+/H^+ exchanger activity in the proximal straight tubule of neonatal rabbits (29) and to increase transcription and mRNA levels for the NHE3 isoform of the protein (29). Third, several studies have directly implicated thyroid hormone and the thyroid hormone receptor in regulation of NHE1 expression. For example, we previously found that thyroid hormone affects endogenous NHE1 message levels in rat hearts (14). In addition, we have shown that the −841/−800 nt region of the NHE1 promoter competes with the palindromic, thyroid receptor-binding DNA sequence in binding of proteins in nuclear extracts (19).

In this study, we confirmed that thyroid hormone levels are important in NHE1 expression using two separate models: isolated myocytes and hearts from hypothyroid, euthyroid, and hyperthyroid rats. Previous experiments (14) have demonstrated that T3 levels affect production of mRNA for NHE1 and that they affect resting intracellular pH. Here, we showed that T3 levels also affect amounts of NHE1 protein in cardiac tissue.

To elicit its physiological effects, T3 binds to cytosolic thyroid receptors, which then bind to specific nucleotide sequences (thyroid hormone response elements) (30). To determine how thyroid hormone affects expression of NHE1, we examined the Na^+/H^+ exchanger promoter. The −841/−800 nt element of this promoter is critical in basal and serum-stimulated regulation of NHE1 expression. Our current experiments with *in vitro* translated TRα confirm that this nuclear hormone receptor binds directly to the −841/−800 nt element of the Na^+/H^+ exchanger promoter. Although TRα binds to a similar region of the element as COUP-TF, significant differences in their binding patterns were observed when we looked at binding to mutant forms of the element. For example, COUP-TF did not bind to M2. In contrast, TRα did bind to M2, with an altered pattern, and it appeared also to bind as a dimer on this mutant.

To better characterize COUP-TF and TR binding to the −841/−800 nt element of the Na^+/H^+ exchanger promoter, we investigated whether these two receptors compete for binding. Decreasing amounts of TR were tested for binding on the element alone or after co-incubation with a constant amount of COUP-TFI. This resulted in less COUP-TF binding and no variation in TR binding. Conversely, when decreasing amounts of COUP-TF were tested for binding, alone or with constant amounts of TR, we again noted that COUP-TF binding was decreased, and TR binding was unchanged. These findings suggest that TR can compete with COUP-TFI for binding to the same or to an overlapping, site. We have shown that COUP-TFs bind to nucleotides −829 to −824 of the 841/−800 nt element (Ref. 19 and Fig. 3). Our current data suggest that this region of the promoter must overlap with the TR-binding site. Because TR binds to the element when this region is mutated yet does not bind when the −838 to −832 region is mutated, it is possible that the primary binding site of TR is nt −838 to −832 and that, when bound, the TR protein overlaps nt −829 to −824.

Our results also suggest that TR binding may be quite promiscuous within the −841/−800 element, in agreement with its already well known plasticity (30). TR can accommodate a multitude of arrangements within its DNA-binding sites. It has been reported to bind as a monomer, homodimer, and/or heterodimer. For TR to bind as a monomer, only one nuclear hormone receptor half-site is necessary. The optimized consensus for the half-site binding motif, (T/C)(A/G)AGGTCA is an octamer that includes a 5′-stabilizing extension (32). Nucleotides −829 to −822 (TGACCTCA, the unmutated M2 region) form a perfect consensus site (TGAGGTCA on the opposite strand). However, because mutation of this region does not eliminate binding of TRα, it is clear that it plays only a partial role, at best, in providing a binding site for TRα. The unmutated M1 region contains a partial, imperfect half-site consensus for the smaller nuclear hormone receptor-binding sequence AGG(T/A)CA that can bind TR (30) from nucleotides −838 to −832, TCTCCCT (AGGAGA, on the opposite strand). Thus a perfect consensus sequence for TR binding is followed by a partial consensus sequence for TR binding, with a 2-base pair spacer. This kind of arrangement (two consensus sequences separated by a spacer) functions as a T3 response element in other systems, modulating transcriptional responses to T3 by malic enzyme (33) and myelin basic protein (Ref. 34; reviewed in Ref. 30). TR can bind to hormone-responsive elements as a monomer, homodimer, or heterodimer (30). In this study the TR appeared to bind as a monomer, although some potential for dimerization was apparent when we looked at binding to mutated elements.

Our results clearly demonstrate that the TRα nuclear receptor can activate the NHE1 promoter through interaction with the −841/−800 nt element. We found that the −841/−800 nt element directed increased transcription of the luciferase gene in response to transfection with the TRα receptor. In contrast, more distal regions of the promoter were not responsive to the expression of TRα. These results, along with *in vivo* observations of the effects of thyroid hormone on NHE1 expression, strongly suggest that T3 activates the NHE1 promoter by this mechanism *in vivo*. In support of this argument were the results showing that T3-treated primary cultures of isolated myocytes bind much more to the −841/−800 nt region of the NHE1 promoter than untreated myocytes. The physiological significance of this regulation has still to be determined. We have recently shown in mice (35) that, in several tissues, the expression of NHE1 initially increases following birth. The protein levels then decline slightly with time. A similar time course of expression has been demonstrated for thyroid hormone in postnatal rats (36), supporting the suggestion that T3 may be responsible for the changes in NHE1 levels that we observed.

Although it is clear that variations in thyroid hormone levels

affect NHE1 expression in the myocardium (Ref. 14 and the present study), it is apparent that this is not a general phenomenon in all tissues. For example, NHE1 message levels in the rat renal cortex are not affected by alterations in thyroid status (37). Earlier studies have demonstrated effects of thyroid hormones on Na^+/H^+ exchanger activity in the kidney (38), but in this study the isoform of the exchanger was not specified and was likely not NHE1 but rather NHE3, which is known to be regulated by thyroid hormone in the kidney (39).

In this study we demonstrated the effects of thyroid hormone in the myocardium. In addition, we found that TRα increased transcription from the NHE1 promoter in fibroblasts and in CV1 cells, a cell line commonly used for expression of nuclear hormone receptors (8). Specificity in the action of TRα is mediated by altering partners in heterodimerization or by associating with additional mediators of transcription, including transcriptional co-activators and repressors (30). Several molecules have been shown to associate with TRα, including RXR and peroxisome proliferator-activated receptor, and thereby modify its nuclear regulatory role (30). The tissue distribution of TRα also varies, possibly accounting for differences in mediation of T3 effects between tissues (31). It is possible, even likely, that the effects of T3 in regulating expression of the Na^+/H^+ exchanger vary greatly from one tissue to another. Future studies may examine this possibility.

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