

Characterization of a Negative Thyroid Hormone Response Element in the Rat Sodium, Potassium-Adenosine Triphosphatase $\alpha 3$ Gene Promoter*

SHING CHIN, JAMES APRILETTI, AND GREGORY GICK

Department of Biochemistry, State University of New York Health Science Center, Brooklyn, New York 11203; and Metabolic Research Unit, University of California (J.A.), San Francisco, California 94143

ABSTRACT

The thyroid hormone L-T₃ elicits either a stimulatory or an inhibitory effect on expression of the Na,K-adenosine triphosphatase $\alpha 3$ -subunit gene in primary cultures of neonatal rat cardiac myocytes. The present study was undertaken to characterize a negative thyroid hormone response element present within the rat Na,K-adenosine triphosphatase $\alpha 3$ -subunit gene proximal promoter. Transient transfection assays indicated that the DNA-binding domain of thyroid hormone receptor was essential for mediating repression of $\alpha 3$ gene transcription by thyroid hormone. This negative effect of thyroid hormone was enhanced in the presence of cotransfected retinoid X

receptor and its ligand 9-*cis*-retinoic acid. Inhibition of $\alpha 3$ chimeric gene expression by thyroid hormone was dependent on the initial cell plating density. The negative thyroid hormone response element was localized to a region between nucleotides -68 to -6 of the $\alpha 3$ gene. Electrophoretic mobility shift assays showed that thyroid hormone receptor binds in a synergistic manner as a heterodimer with retinoid X receptor to two sites at positions -62 to -41 and -39 to -17 of the $\alpha 3$ gene promoter. The upstream and downstream heterodimer binding sites coexist with CAAT and TATA elements, respectively. (*Endocrinology* **139**: 3423-3431, 1998)

Na,K-ADENOSINE triphosphatase (Na,K-ATPase) is a membrane-associated enzyme that catalyzes transport of Na⁺ and K⁺ ions across the plasma membrane of all animal cells (1). The activity of Na,K-ATPase is integral for the establishment of an electrochemical gradient, which is essential for maintaining the resting potential, cell volume, and osmotic balance (2). As the only known receptor for cardiac glycosides such as ouabain and digitalis, Na,K-ATPase is a target for treatment of congestive heart failure and cardiac arrhythmias (3). Na,K-ATPase consists of two subunits, α and β , and each subunit has several isoforms (4). Expression of α isoform genes $\alpha 1$, $\alpha 2$, and $\alpha 3$ is tissue specific. In the adult rat, for instance, $\alpha 1$ subunit expression is nearly constitutive, whereas the $\alpha 2$ isoform is mainly present in neural and muscle tissue. Na,K-ATPase $\alpha 3$ -subunit is limited primarily to neural tissues. Major changes in Na,K-ATPase α isoform content also occur during development. For example, $\alpha 1$ is constitutively expressed throughout development in rat heart. In contrast, $\alpha 2$ is predominantly found in adult heart, whereas $\alpha 3$ is selectively up-regulated in fetal and neonatal myocardium (5). In addition, each α isoform exhibits differential ouabain sensitivity and affinity for Na⁺ ions (6).

Regulation of Na,K-ATPase activity and subunit gene expression has been of great interest because of its vital phys-

iological and therapeutic significance. L-T₃ is the major active form of thyroid hormone and stimulates Na,K-ATPase activity and isoform messenger RNA (mRNA) content in a variety of mammalian tissues (7-9). Both transcriptional and posttranscriptional mechanisms have been implicated in the regulation of Na,K-ATPase subunit gene expression by T₃ (8, 10-13). T₃ regulation of Na,K-ATPase $\alpha 3$ subunit gene expression has been studied in primary cultures of neonatal rat cardiac myocytes. In this cell culture system, Kamitani *et al.* (11) observed a 3-fold stimulation of $\alpha 3$ mRNA content by T₃ at 1 day and a 50% decrease with prolonged exposure to hormone. Furthermore, in this study, a 2.6-kb (-2500/+136) portion of the $\alpha 3$ gene conferred a 3-fold stimulation of reporter gene expression in transient transfection assays (11). In contrast, we found that the region between nucleotides -116 to -6 of the $\alpha 3$ gene promoter suppressed chimeric gene expression about 50% in primary cultures of cardiac myocytes incubated in the presence of T₃ and cotransfected T₃ receptor (T₃R) expression vector (14).

T₃ exerts its transcriptional function through an interaction with T₃R, which is preassociated with a specific DNA sequence, a T₃ response element (TRE). A hexanucleotide half-site, AGGTCA, has been identified as a consensus sequence for binding of T₃R (15). Naturally occurring TREs usually are arranged as direct repeats (DRs) of a half-site, a palindrome, or an inverted palindrome (16-18). Interestingly, DRs with different spacings are also recognized by retinoic acid receptor (RAR), vitamin D receptor, and retinoid X receptor (RXR), which, along with T₃R, comprise a nuclear receptor superfamily (19). In addition, RXR is capable of enhancing DNA binding of T₃R, RAR, and vitamin D receptor by formation of a heterodimer (20, 21). The effect of T₃ on gene transcription can be either stimulatory or inhibitory, depend-

Received January 12, 1998.

Address all correspondence and requests for reprints to: Gregory Gick, Ph.D., Department of Biochemistry, State University of New York Health Science Center, Brooklyn, New York 11203. E-mail: gickg11@hscbklyn.edu.

* This work was supported by grants from the National Science Foundation and an Investigatorship and Grant-in-Aid from the American Heart Association, New York City Affiliate (to G.G.), and Grant DK-41842 (to J.A.).

ing on the cell type and the specific sequence and arrangement of the individual TRE (22). A palindrome of AGGTCA or DRs with a four-nucleotide spacing has been widely accepted as a positive TRE (pTRE) (22), whereas the nature of a negative TRE (nTRE) has not been well defined.

In a previous study we identified a nTRE in the -116 to -6 bp region of the rat Na,K-ATPase $\alpha 3$ -subunit gene (14). To continue our characterization of this nTRE, transient transfection assays were conducted to evaluate the molecular mechanism underlying T_3 -mediated repression of $\alpha 3$ gene transcription. T_3 R binding sites within the $\alpha 3$ gene proximal promoter region were localized by electrophoretic mobility shift assays (EMSAs). We report here the identification of two binding sites for T_3 R/RXR heterodimers within a 63-bp region of the $\alpha 3$ gene promoter containing a functional nTRE.

Materials and Methods

Cell culture

Neonatal rat cardiac myocytes were prepared by a trypsin/deoxyribonuclease I digestion method as previously described (13). Cells were maintained for 1 day in DMEM containing 7% FBS and antibiotics (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 250 ng/ml amphotericin B as fungizone). After transfection on day 2 of culture, cardiac myocytes were incubated in serum-free DMEM supplemented with 10 μ g/ml insulin, 10 μ g/ml transferrin, and antibiotics for 1 day and then treated with T_3 and/or 9-*cis*-retinoic acid (9-*cis*-RA).

Construction of $\alpha 3$ /luciferase chimeric genes

pLUC36 is an $\alpha 3$ /luciferase gene construct containing $\alpha 3$ sequence spanning nucleotides -116 to -6 in a promoterless firefly luciferase plasmid pXP1 (14). Two additional deletion constructs, pLUC38 and pLUC39, which contain the $\alpha 3$ gene $-68/-6$ and $-39/-6$ regions, respectively, were derived from pLUC36. To generate pLUC38, pLUC36 was digested with *Bsr*BI and *Hind*III to release a fragment containing the $\alpha 3$ gene $-68/-6$ region, which was subsequently ligated with *Sma*I- and *Hind*III-digested pXP1. To generate pLUC39, pLUC36 was digested with *Sac*I and *Sac*II to remove the $\alpha 3$ gene $-116/-40$ region. The remaining fragment containing nucleotides between -39 and -6 within the pXP1 vector was treated with T4 polymerase to produce blunt ends and then religated with T4 ligase. These constructs were subjected to both restriction enzyme digestion and double stranded DNA sequencing.

Transient transfection

Cardiac myocytes (4.0×10^6 cells/6-cm plate) were transfected via a calcium phosphate coprecipitation method as previously described (13). For T_3 studies, 5.0 μ g pLUC36, pLUC38, or pLUC39 were cotransfected with 5.0 μ g of either a rat T_3 R $\beta 1$ expression vector (23) or a mutant rat T_3 R $\beta 1$ expression plasmid that has an internal deletion of the DNA-binding domain at amino acids 100–171 (24). To assess the effect of cell density on T_3 -mediated repression, pLUC36 and T_3 R $\beta 1$ plasmids were cotransfected into cells at both 4.0 and 8.0×10^6 cells/6-cm plate. For RXR cotransfection studies, cells were cotransfected with 2.0 μ g of either pLUC36 or positive retinoid X response element (pRXRE)/LUC and 2 μ g of a mouse RXR β expression vector (25). pRXRE/LUC is a luciferase reporter gene containing three pRXREs in front of the thymidine kinase promoter (26). For activating protein-2 (AP2) and Sp1 cotransfection studies, cells were transfected with 2 μ g pLUC36 along with 2 μ g of either pRSVAP2, an AP2 expression vector (27), or pPACSP1, an Sp1 expression vector (28). Cells were treated with 100 nM T_3 , 1 μ M 9-*cis*-RA, or both for 1 day. Cells were lysed by incubation for 15 min at room temperature with 200 μ l reporter lysis buffer (Promega, Madison, WI) containing 1% Triton-X 100, 10% glycerol, 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, and 20 mM dithiothreitol (DTT). To measure luciferase activity, 25 μ l of cell lysate were combined with 100 μ l substrate [470 μ M luciferin, 270 μ M coenzyme A, 530 μ M ATP, 20 mM tricine (pH 7.8), 1.1 mM (MgCO₃)₄ Mg(OH)₂·5H₂O, 2.7 mM MgSO₄, 0.1 mM EDTA, and 33.3 mM

DTT], and photon emission was counted in a liquid scintillation counter within 20 sec. Luciferase activity was expressed per total cellular protein as previously described (14).

Bacterial expression and purification of receptors

Escherichia coli-expressed human T_3 R $\beta 1$ was purified to approximately 7% homogeneity by phenol hydrophobic interaction chromatography and heparin affinity chromatography steps (29). Plasmids pGEXKG-rRXR β and pGEXKG-hT₃R $\beta 1$ express rat RXR β -glutathione-S-transferase (GST) and human T_3 R $\beta 1$ -GST fusion proteins, respectively (20). These plasmids were transformed into bacterial strain JM 83. GST fusion protein was prepared according to a protocol provided by Pharmacia (Piscataway, NJ). Briefly, the recombinants were grown at 37 C in 500 ml Luria Beroni medium containing ampicillin until an OD at 600 nm of 0.8–1.0 was reached. Cells were induced by incubation with 0.5 mM isopropylthiogalactoside for 3 h, centrifuged, and lysed by three cycles of sonication for 30 sec each. Supernatants of cell lysates were incubated with 1 ml glutathione agarose beads [50% (vol/vol) in 1 \times PBS and 1% Triton-X 100] with slow shaking at 4 C for 30 min. Finally, GST fusion protein was eluted by resuspension of the beads in 1 ml of a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM glutathione, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol and stored at -70 C. The purity of GST fusion proteins was assessed by SDS-PAGE, and the concentration was estimated by determination of OD at 600 nm.

EMSAs

Oligonucleotides that span the $\alpha 3$ gene regions $-116/-72$, $-62/-21$, $-62/-41$, $-44/-21$, $-39/-17$, and $-28/-6$ were synthesized, as were mutation-containing $-62/-41$ and $-39/-6$ oligonucleotides (Life Technologies, Grand Island, NY). The fragments spanning the $-116/-6$ and $-39/-6$ regions were generated by digestion of pUC19($-116/-6$), which contains the -116 to -6 bp region of the $\alpha 3$ gene with *Pvu*III/*Xba*III and *Sac*II/*Xba*III, respectively. DNA was labeled as previously described (13). Briefly, 50 ng of either annealed oligonucleotide or DNA fragments produced by restriction enzyme digestion were incubated with 6 U Klenow fragment in the presence of 1 mM deoxy (d)-ATP, dTTP, and dGTP and 50 μ Ci [³²P]dCTP (3000 Ci/mmol) at room temperature for 20 min. Unincorporated [³²P]dCTP was removed by chromatography on Sephadex G-25 spin columns. To detect T_3 R and RXR binding to $\alpha 3$ gene fragments in a 15- μ l reaction, each labeled DNA (2.0 – 5.0×10^5 cpm, 1.0 ng) was incubated with 6 ng human T_3 R $\beta 1$ or 0.4 μ g human T_3 R $\beta 1$ -GST and/or 1.3 μ g rat RXR β -GST in the presence of 1.5 μ g polyd(I-C), 1 mM DTT, 10% glycerol, 10 mM HEPES (pH 7.9), 75 mM KCl, and 1 mM EDTA for 20 min at room temperature. For competition experiments, unlabeled 10-, 100-, or 1000-fold excesses of wild-type or mutant $-62/-41$ oligonucleotides were incubated with T_3 R $\beta 1$ and RXR β for 10 min before addition of labeled wild-type $-62/-41$ oligonucleotide. The reaction was continued for another 20 min. Electrophoresis in 5–8% polyacrylamide gels was carried out under low ionic strength conditions ($0.5 \times$ TBE) at 4 C. Gels were dried and either exposed to x-ray film at -70 C or subjected to phosphorimaging analysis.

Statistical analysis

Results are expressed as the mean \pm SEM. Statistical significance was determined by unpaired Student's *t* test (two tailed).

Results

DNA-binding domain of T_3 R and lower cell density are required for T_3 -mediated repression of $\alpha 3$ gene transcription

Both ligand- and DNA-binding domains of T_3 R are essential for mediating the stimulatory transcriptional effect of T_3 (22); however, the role of binding of T_3 R to nTREs is less well defined. To examine this issue, a mutant T_3 R $\beta 1$ expression vector that has its DNA-binding domain deleted was cotransfected with $\alpha 3$ /luciferase gene construct pLUC36 ($-116/-6$ bp) in transient transfection experiments with primary cultures of neonatal rat cardiac myocytes. As shown

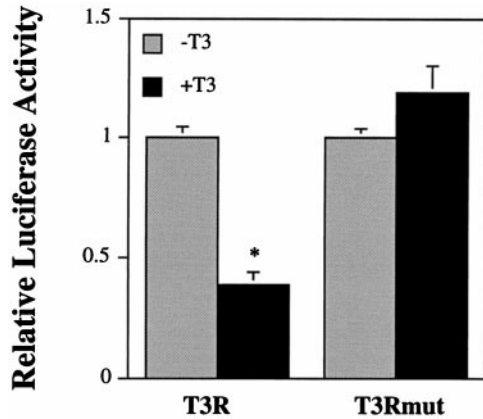


FIG. 1. DNA-binding domain of $T_3R\beta 1$ is required for T_3 -mediated repression of $\alpha 3$ gene transcription. Neonatal rat cardiac myocytes were maintained under conditions defined in *Materials and Methods*. pLUC36 (2 μ g) was cotransfected with equivalent amounts of either a wild-type or a mutant $T_3R\beta 1$ (T_3Rmut) expression vector. The mutant $T_3R\beta 1$ has a deletion of its DNA-binding domain. After treatment with 100 nM T_3 for 1 day, cells were lysed, and luciferase activity was measured and normalized to protein content. Data (n = 6) are presented as the ratio of the normalized luciferase activity in the presence of T_3 relative to that in the absence T_3 , which is set at 1.0. *, $P < 0.05$, with T_3 vs. without T_3 .

in Fig. 1, pLUC36 was repressed 60% by T_3 in the presence of wild-type $T_3R\beta 1$. In the presence of cotransfected mutant $T_3R\beta 1$, however, T_3 did not repress pLUC36 expression. This indicated that DNA binding of $T_3R\beta 1$ is required for T_3 -mediated repression of $\alpha 3$ gene transcription.

As cell to cell interactions have been implicated as important parameters in hormone responsiveness (30, 31), we evaluated the effect of cell density on T_3 -mediated inhibition of $\alpha 3$ gene transcription (Fig. 2). Neonatal rat cardiac myocytes were plated at two different densities and transfected with pLUC36 and a $T_3R\beta 1$ expression vector. At a lower cell density (4×10^6 cells/6-cm plate), pLUC36 conferred a T_3 -mediated repression of 60%. By contrast, T_3 had no effect on pLUC36 expression when cardiac myocytes were plated at a higher cell density (8×10^6 cells/6-cm plate).

Effect of RXR on T_3 -mediated inhibition of $\alpha 3$ gene expression

Our previous *in vitro* binding studies demonstrated that RXR β was capable of producing a synergistic increase in binding of $T_3R\beta 1$ to the $\alpha 3$ gene region between nucleotides -116 and -6, which contained a functional nTRE (14). It was intriguing to ask whether a similar synergistic effect of $T_3R\beta 1$ and RXR β could be observed *in vivo*. To this end, neonatal rat cardiac myocytes were transfected with pLUC36 with or without cotransfection of an equivalent amount of either a $T_3R\beta 1$ or RXR β expression vector, or both. After cells were treated with 100 nM T_3 , 1 μ M 9-*cis*-RA, or both for 1 day, cells were lysed, and luciferase activity was measured (Fig. 3). Consistent with our earlier results (14), $T_3R\beta 1$ in the presence of T_3 suppressed pLUC36 activity 63% (lane 2). Interestingly, a combination of RXR β and 9-*cis*-RA repressed pLUC36 expression 40% (lane 3), indicating that a negative RXRE (nRXRE) is present in the $\alpha 3$ gene -116/-6 region. The transcriptional activity of pLUC36 was further decreased if

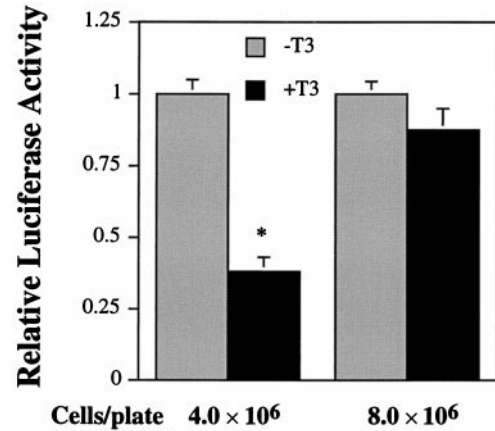


FIG. 2. The effect of cell density on repression of $\alpha 3$ gene promoter activity by T_3 . Neonatal rat cardiac myocytes were cultured at two different cell densities as described in *Materials and Methods*. Cells were transfected with 2 μ g pLUC36 and an equivalent amount of a $T_3R\beta 1$ expression vector. After cells were treated with 100 nM T_3 for 1 day, luciferase activity was measured as in Fig. 1. Data (n = 13–16) are presented as the ratio of luciferase activity in the presence of T_3 relative to that in the absence of T_3 . *, $P < 0.05$, with T_3 vs. without T_3 .

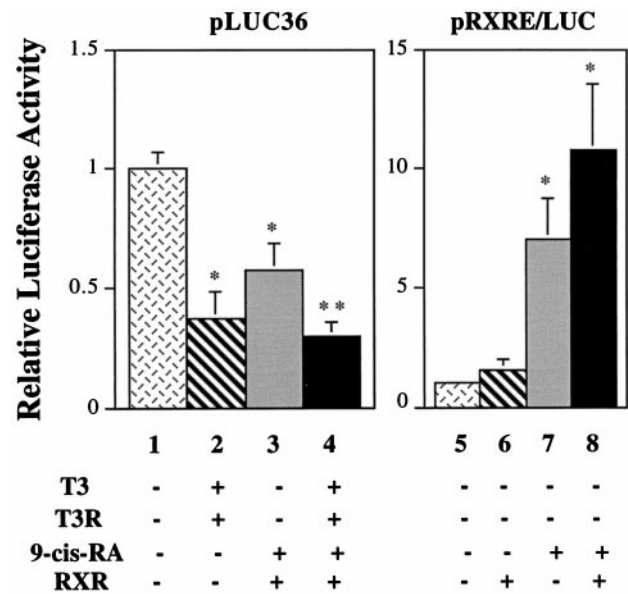


FIG. 3. Role of RXR in T_3 -mediated repression of $\alpha 3$ gene transcription. Cardiac myocytes cultured as described in Fig. 1 were transfected with 2 μ g of either pLUC36 (left) or pRXRE/LUC (right) with or without cotransfection of 2 μ g of either $T_3R\beta 1$ or RXR β expression vectors. Cells were treated with 100 nM T_3 and/or 1 μ M 9-*cis*-RA for 1 day. Luciferase activity was assayed as detailed in Fig. 1. Luciferase activities of pLUC36 and pRXRE/LUC were expressed, respectively, relative to the basal activities of pLUC36 (lane 1) and pRXRE/LUC (lane 5), which were set at 1.0 (n = 10). *, $P < 0.05$; **, $P < 0.05$ (lane 4 vs. 3).

cells were cotransfected with $T_3R\beta 1$ and RXR β and treated with both T_3 and 9-*cis*-RA (lane 4). As a positive control, pRXRE/LUC, containing three pRXREs was stimulated by 9-*cis*-RA in the absence (lane 7) and presence of cotransfected RXR β (lane 8). These observations suggest that a functional interaction may occur between $T_3R\beta 1$ and RXR β in mediat-

ing repression of $\alpha 3$ gene transcription via binding to a nTRE within the proximal promoter.

Neither Sp1 nor AP2 mediates the inhibitory action of T_3 on $\alpha 3$ gene transcription

The transcription factors Sp1 and AP2 have been suggested to play a role in the T_3 responsiveness of several genes (20, 32, 33). As the -116 to -6 bp region of the $\alpha 3$ gene promoter contains potential binding sites for both Sp1 and AP2 (34, 35), we investigated whether either of these *trans*-activators contributes to T_3 -mediated repression of $\alpha 3$ gene expression. To address this issue, pLUC36 expression was evaluated in the presence of either cotransfected Sp1 or AP2 expression vectors. As illustrated in Fig. 4, cotransfection of AP2 did not affect pLUC36 activity (lane 2), whereas Sp1 stimulated pLUC36 expression 2-fold (lane 3). However, neither AP2 nor Sp1 counteracted T_3 -mediated repression of pLUC36 expression (lane 5 *vs.* lane 6 or 7).

nTRE is localized to the $-68/-6$ region of the $\alpha 3$ gene promoter

To further localize the nTRE present within the $-116/-6$ bp region of the $\alpha 3$ gene promoter, we prepared two additional 5'-deletion constructs containing the -68 to -6 bp and -39 to -6 bp regions of the $\alpha 3$ gene. Before analysis of the effect of T_3 , we evaluated the basal activity of these deletion constructs (Fig. 5A). In construct pLUC38, deletion of $\alpha 3$ sequence from -116 to -69 bp lead to an approximately 65% reduction of basal gene transcription compared with the activity of pLUC36. Expression of construct pLUC39 containing 34 bp of 5'-flanking DNA was only marginally higher

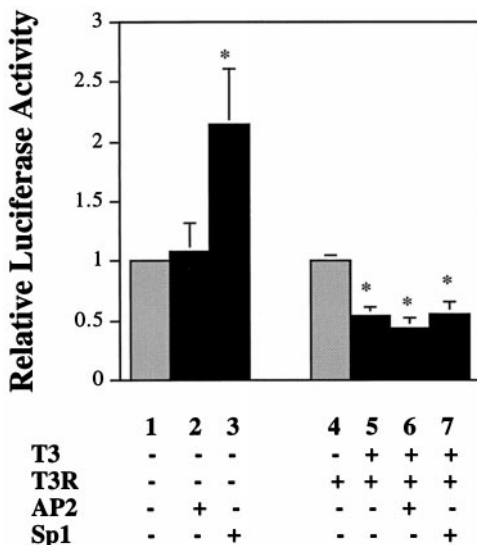


FIG. 4. Effects of AP2 and Sp1 on inhibition of $\alpha 3$ gene promoter activity by T_3 . Neonatal rat cardiac myocytes were maintained as defined in Fig. 1 and transfected with $2 \mu\text{g}$ pLUC36 with or without cotransfection of $2 \mu\text{g}$ of a $T_3R\beta 1$, Sp1, or AP2 expression vector. Cells were exposed to 100 nM T_3 for 1 day, and luciferase activity was quantitated as in Fig. 1. Values of the control groups (lanes 1 and 4) were set at 1.0, and values of experimental groups were expressed relative to that of the control group ($n = 8$). *, $P < 0.05$ (lane 3 *vs.* 1, lane 5, 6, or 7 *vs.* 4).

than the background expression observed in nontransfected cell lysates. Thus, we did not continue our characterization of the nTRE in the $\alpha 3$ gene proximal promoter with the pLUC39 construct. Transient transfection assays were conducted, however, with the new 5'-deletion construct pLUC38 (Fig. 5B). In primary cultures of neonatal rat cardiac myocytes cotransfected with an expression vector encoding $T_3R\beta 1$, expressions of pLUC36 and pLUC38 were repressed 44% and 39%, respectively, in response to T_3 . These data indicate that the region of the $\alpha 3$ promoter between nucleotides -68 and -6 contains a functional nTRE.

T_3R/RXR heterodimers bind to the $-62/-21$ region of the $\alpha 3$ gene

Extensive studies on T_3 -responsive genes have identified a hexanucleotide sequence, AGGTCA, in the form of DRs, inverted repeats, or a palindrome as a pTRE (19). Binding of T_3R as a monomer to this hexanucleotide motif has been implicated in repression of rat TSH gene transcription by T_3 (36). A computer-assisted search of the $\alpha 3$ gene promoter between nucleotides -68 to -6 revealed three regions, located at $-60/-55$, $-34/-29$, and $-20/-15$ bp, with a four of six match to the AGGTCA motif (Fig. 6).

To determine whether the three potential half-sites are indeed T_3R -binding sites, EMSAs were performed using DNA fragments that span the subregion of the $\alpha 3$ gene promoter from -116 to -6 bps (Fig. 7). The $\alpha 3$ gene regions $-116/-6$, $-116/-72$, and $-62/-21$ were labeled with $\alpha^{32}\text{P}$ dCTP and incubated with $T_3R\beta 1$, $RXR\beta$, or both receptors. Samples were resolved by 5% PAGE, and DNA-protein complexes were detected by autoradiography (Fig. 7A). In the presence of $T_3R\beta 1$ alone, weak binding of T_3R monomer was detected to the $\alpha 3$ region $-62/-21$ (lane 7), consistent with our earlier observation (14) (lane 1). In the presence of both $T_3R\beta 1$ and $RXR\beta$, however, strong binding was observed with the $\alpha 3$ gene $-62/-21$ region, indicating the formation of a $T_3R\beta 1/RXR\beta$ heterodimer (lane 9). By contrast, heterodimer binding was not evident in the $\alpha 3$ gene fragment $-116/-72$. Therefore, a specific binding site for $T_3R\beta 1/RXR\beta$ heterodimers is present in the $-62/-21$ region of the $\alpha 3$ gene.

Oligonucleotides spanning nucleotides -62 to -41 and -44 to -21 were used in EMSAs to further localize $T_3R\beta 1$ and $RXR\beta$ binding within the region of the $\alpha 3$ gene containing a nTRE (Fig. 7B). $T_3R\beta 1$ associated with $RXR\beta$ -GST and formed complexes with the $-62/-41$ oligonucleotide (lanes 7 and 8), but did not bind to the $-44/-21$ oligonucleotide (lanes 11 and 12). A selective effect of $T_3R\beta 1$ is indicated by the observation that both native $T_3R\beta 1$ and $T_3R\beta 1$ -GST proteins participated in heterodimer formation with RXR . In addition, there was an approximately 25-fold decrease in the intensity of $T_3R\beta 1/RXR\beta$ binding to the $-62/-41$ oligonucleotide compared with that of the $-62/-21$ region probe (lane 3 *vs.* 7 and lane 4 *vs.* 8).

To examine whether the potential T_3R binding site between nucleotides -60 and -55 (Fig. 6) was indeed associated with $T_3R\beta 1/RXR\beta$ binding to the $-62/-41$ oligonucleotide, mutations were produced at three positions that are homologous to the T_3R -binding site hexamer AGGTCA. Both

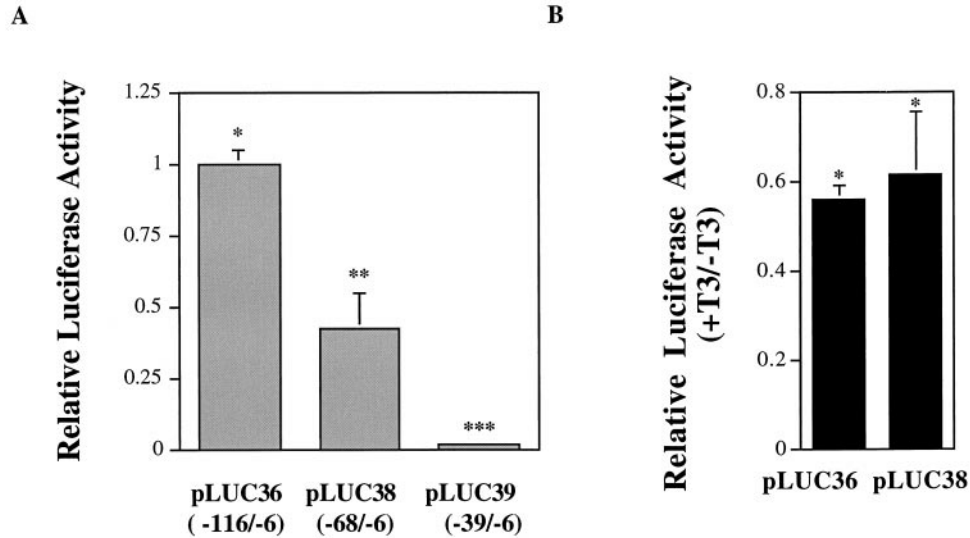


FIG. 5. A nTRE exists in the -68 to -6 bp region of the $\alpha 3$ gene promoter. A, Primary cultures of cardiac myocytes were incubated as described in Fig. 1 and transfected with $5 \mu\text{g}$ of pLUC36, pLUC38, or pLUC39 in the presence of $5 \mu\text{g}$ cotransfected T₃R β 1 expression vector. Luciferase activity associated with pLUC36 was set at 1.0, and the activities of pLUC38 and pLUC39 were expressed relative to pLUC36 ($n = 8-10$). The promoter activity of any of these three constructs is statistically significantly different from the other two, as indicated by asterisks ($P < 0.05$). B, Cardiac myocytes cultured as described in Fig. 1 were transfected with $5 \mu\text{g}$ of either pLUC36 or pLUC38 and $5 \mu\text{g}$ of a T₃R β 1 expression vector. After treatment with 100 nM T₃ for 1 day, cells were lysed, and luciferase activity was measured as described in Fig. 1. Data ($n = 8-14$) are presented as the ratio of the normalized luciferase activity in the presence of T₃ relative to that in the absence of T₃. *, $P < 0.05$, with T₃/without T₃.

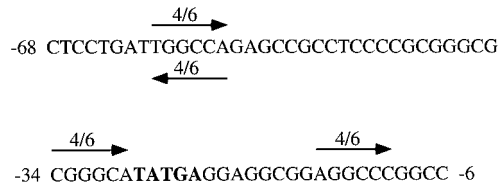


FIG. 6. Potential T₃R-binding sites in the $-68/-6$ region of the $\alpha 3$ gene. Sequences with a four of six match to the T₃R binding site consensus AGGTCA motif are indicated by *overhead arrows*. The TATA box is in *boldface*.

wild-type and mutation-containing $-62/-41$ oligonucleotides were tested for binding of T₃R β 1/RXR β in EMSAs (Fig. 8, *left panel*). T₃R β 1/RXR β heterodimer formation was evident only on the wild-type $-62/-41$ oligonucleotide (lane 2 *vs.* 4). The specificity of T₃R β 1/RXR β binding to the $-62/-41$ region was examined in a competition experiment (Fig. 8, *right panel*). A 10-fold excess of the wild-type $-62/-41$ oligonucleotide competitor did not reduce T₃R β 1/RXR β formation (lane 6); however, a 100-fold excess of wild-type competitor lead to a 69% decrease in binding of heterodimers (lane 7), and a 1000-fold excess of unlabeled wild-type oligonucleotide abolished binding (lane 8). In contrast, the mutant $-62/-41$ oligonucleotide did not compete efficiently (lanes 9–11). These observations indicate that T₃R β 1/RXR β heterodimers bind in a specific manner to the -62 to -41 bp region of the $\alpha 3$ gene.

The striking difference in the apparent binding affinity of T₃R β 1/RXR β to oligonucleotides comprising the $-62/-21$ and $-62/-41$ regions (Fig. 7B) raised the possibility that an additional T₃R β 1/RXR β binding site(s) is present downstream of the $-62/-41$ region. Indeed, two regions exist between nucleotides -34 and -29 and -20 and -15 with

partial homology to the consensus T₃R binding motif AGGTCA (Fig. 6). To test this hypothesis, a fragment between nucleotides -39 to -6 of the $\alpha 3$ gene was labeled and examined in EMSAs (Fig. 9). Binding of T₃R β 1/RXR β heterodimers to the -39 to -6 bp region was detected (lane 4). Heterodimer formation was also evident in EMSAs using an oligonucleotide spanning the -39 to -17 region (lane 12). In contrast, no binding was detected to an oligonucleotide containing $\alpha 3$ sequence from -28 to -6 bps (lane 16). Mutation of four conserved nucleotides within the potential T₃R-binding site between -34 and -29 bp abolished heterodimer binding (lane 8). Taken together, these data suggest that a second T₃R β 1/RXR β -binding site exists within the proximal promoter of the $\alpha 3$ gene.

Discussion

Our earlier studies identified a nTRE in the region of the rat Na,K-ATPase $\alpha 3$ gene between nucleotides -116 and -6 (14). In this report, we have characterized functional aspects of this nTRE and have defined binding sites for T₃R β 1 and RXR β . The results indicate that the DNA-binding domain of T₃R β 1 was necessary to produce repression of $\alpha 3$ gene transcription. Similarly, T₃-mediated inhibition of transcription of the epidermal growth factor receptor gene was abrogated in the presence of the identical mutant T₃R β 1 used in our studies (24). In contrast, repression of GHF-1/Pit-1 gene expression by T₃ did not require direct DNA binding of T₃R (37). Instead, protein-protein interactions between T₃R and a cAMP-responsive element-binding protein were postulated to contribute to T₃-mediated repression.

Repression of $\alpha 3$ gene transcription by T₃ was abolished at a high cell density, which yielded an approximately 70% confluent culture of neonatal rat cardiac myocytes. To our

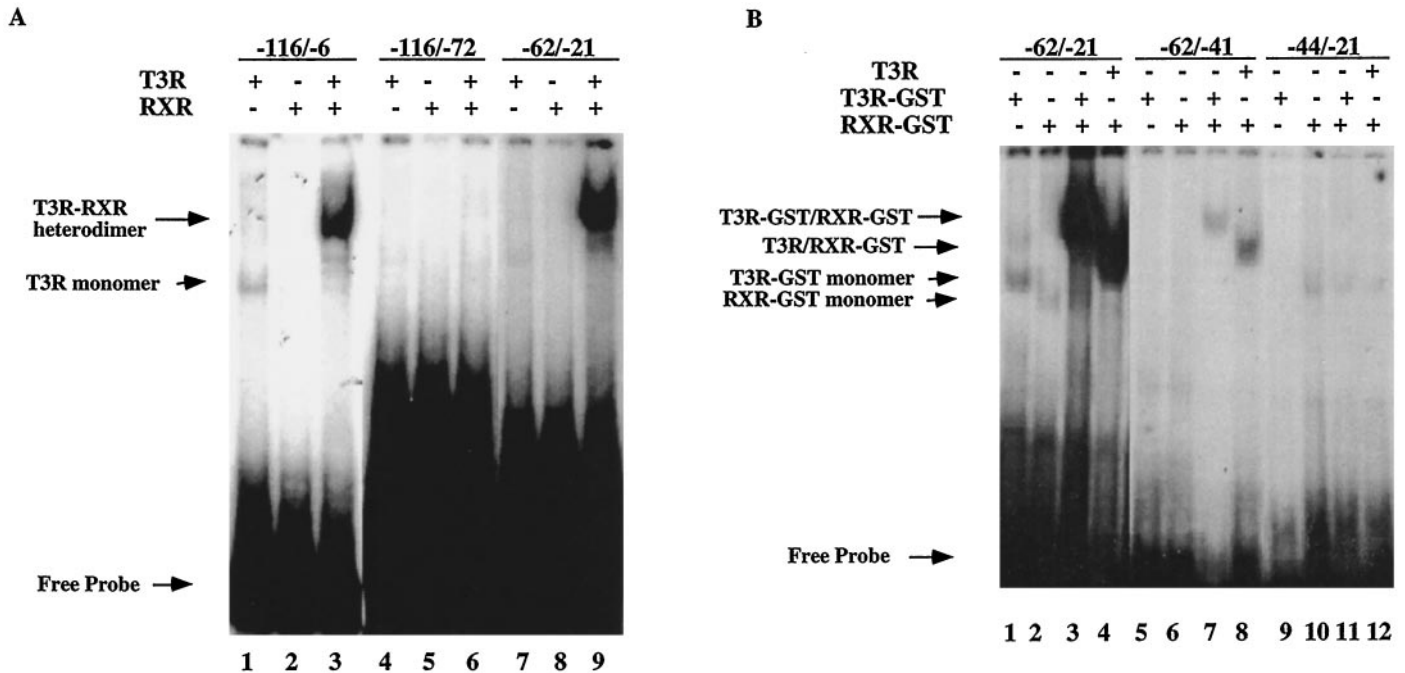


FIG. 7. Formation of $T_3R\beta 1/RXR\beta$ heterodimers on the $\alpha 3$ gene $-62/-21$ region. A, $\alpha 3$ gene fragments $-116/-6$, $-116/-72$, and $-62/-21$ were labeled with [^{32}P]dCTP. Probes were incubated with $T_3R\beta 1$, $RXR\beta$ -GST, or both receptors as described in *Materials and Methods*. Electrophoresis was carried out in a 5% polyacrylamide gel under lower ionic strength ($0.5 \times$ TBE) at 15 mA. This result is representative of three experiments. B, ^{32}P -Labeled $-62/-21$, $-62/-41$, and $-44/-21$ regions of the $\alpha 3$ gene were incubated with either $T_3R\beta 1$ or $T_3R\beta 1$ -GST with or without $RXR\beta$ -GST. The reaction products were resolved using 6% PAGE, and DNA-protein complexes were detected by autoradiography. This result is representative of three experiments.

knowledge, this is the first demonstration that cell density influences T_3 responsiveness of a mammalian gene. Our findings are consistent with previous observations that an alteration of cell density may affect the responsiveness of cells to other hormones (30, 31). For example, 17β -estradiol increased PRL mRNA levels in low and intermediate density cultures of pituitary tumor GH_4C_1 cells, whereas it did not affect PRL mRNA content in high density cultures (30). In contrast, clone 5 mRNA was inducible by glucocorticoid hormone only in a high density culture of adipogenic TA1 cells (31). Although the molecular events associated with loss of T_3 -mediated repression of $\alpha 3$ gene transcription at high cell density have not been delineated, we speculate that the underlying mechanism may involve either the cell density-dependent activation or accumulation of an antagonist of T_3R .

As we previously demonstrated a synergistic effect of $RXR\beta$ on $T_3R\beta 1$ binding to the nTRE located between nucleotides -116 and -6 of the $\alpha 3$ gene (14), we initiated an investigation of the functional effect of $RXR\beta$ on T_3 -mediated repression of $\alpha 3$ gene transcription. Cotransfection of $T_3R\beta 1$ and $RXR\beta$ in the presence of T_3 and 9 -cis-RA yielded the greatest degree of repression of $\alpha 3$ chimeric gene expression, and the difference was statistically significant compared with the inhibition seen in the presence of $RXR\beta$ and its ligand. These results suggest that a functional interaction between $T_3R\beta 1$ and $RXR\beta$ may occur in the *in vivo* regulation of $\alpha 3$ gene transcription. This finding is also consistent with the results reported by Hollenberg *et al.* (38) in their examination of the nTRE present in the human TRH gene. The functional

response of nTREs to RXR is not uniform, as the inhibitory effect of T_3 on rat and mouse $TSH\beta$ gene transcription was abrogated in the presence of cotransfected RXR and exposure to 9 -cis-RA (39).

Interestingly, we observed a T_3R -independent inhibitory effect of $RXR\beta$ and 9 -cis-RA on $\alpha 3$ transcription in transient transfection studies, suggesting that a nRXRE exists within the -116 to -6 bp region of the $\alpha 3$ gene. Consistent with this potential functional role of $RXR\beta$, we detected binding of $RXR\beta$ to nucleotides between -62 to -21 . The suppression of murine $TSH\beta$ promoter activity by 9 -cis-RA and the thyrotrope-specific $\gamma 1$ isoform of RXR represents the only other example of RXR -mediated repression of gene transcription (40). In this study a nRXRE was localized to nucleotides between -200 and -149 of the $TSH\beta$ gene promoter region, distant from the known $TSH\beta$ nTRE, which is found close to the transcription start site. Whether the $\alpha 3$ gene nRXRE is colocalized or distinct from the nTRE will require further investigation.

Our data indicate that the $\alpha 3$ gene nTRE is localized to a region of the proximal promoter between nucleotides -68 to -6 . Within this 63-bp region, there exist two specific $T_3R\beta 1/RXR\beta$ -binding sites between nucleotides -62 and -41 and nucleotides -39 and -17 . To determine whether one or both of these receptor binding sites were necessary for T_3 -mediated repression of $\alpha 3$ gene transcription, we prepared a deletion construct containing nucleotides between -39 to -6 of the $\alpha 3$ gene promoter and synthesized chimeric constructs with mutations that abolished $T_3R\beta 1/RXR\beta$ heterodimer binding at both sites (data not shown). Unfortunately, both

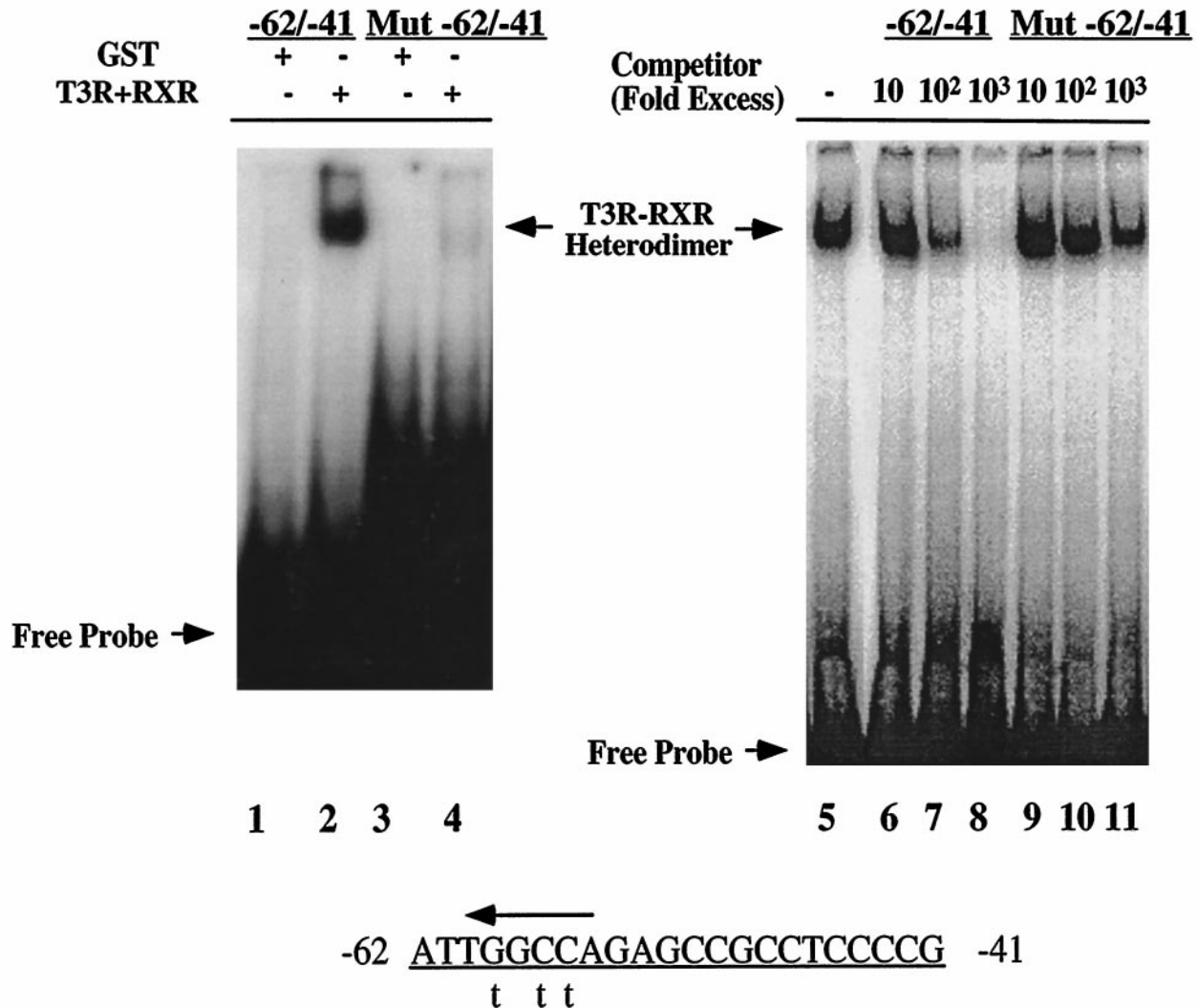


FIG. 8. $T_3R\beta 1/RXR\beta$ heterodimers bind to a site within the $-62/-41$ region of the $\alpha 3$ gene. In the *left panel*, ^{32}P -labeled $\alpha 3$ gene oligonucleotide $-62/-41$ and mutant $-62/-41$ were incubated with either GST or $T_3R\beta 1$ and $RXR\beta$ -GST as indicated in Fig. 7. Three nucleotides were changed (*lower case*) in the potential T_3R -binding site (*arrow*) present in the complementary strand that has a four of six match to the AGGTCA motif to generate the mutant $-62/-41$ region (mut $-62/-41$). In the *right panel*, unlabeled oligonucleotides $-62/-41$ or mut $-62/-41$ were incubated with $T_3R\beta 1$ and $RXR\beta$ -GST for 10 min before addition of ^{32}P -labeled $-62/-41$. Reaction samples were resolved by 6% PAGE, and DNA-protein complexes were detected by autoradiography. This result is representative of three experiments.

of these strategies lead to basal luciferase expression that was too low to proceed with further functional characterization of the $\alpha 3$ gene nTRE. It is interesting to note that a deletion of the nTRE-containing region between -244 to -180 bp in the human glycoprotein hormone α gene resulted in a reduction of its basal activity to background levels (41). Similarly, mutations in the two repeats of a composite hormone response element of the human apolipoprotein AI gene that abolished the binding of RAR, RXR, T_3R , and hepatic nuclear factor 4 also abrogated baseline promoter activity (42).

To investigate the molecular mechanism underlying repression of $\alpha 3$ gene transcription by T_3 , we evaluated whether T_3R might interfere with the stimulatory action of either AP2 or Sp1. We found that neither cotransfected AP2 nor Sp1 counteracted the inhibitory effect of T_3 on $\alpha 3$ gene expression. These results suggest that interference between $T_3R\beta 1$ and either AP2 or Sp1 is not likely to account for the

suppression of $\alpha 3$ promoter activity in response to T_3 . In contrast, binding of T_3R and Sp1 to mutually exclusive overlapping sites was implicated in the mechanism of T_3 repression of both epidermal growth factor receptor and human immunodeficiency virus gene expression (24, 43). Given the close proximity of the downstream $T_3R\beta 1/RXR\beta$ -binding site and the TATA element of the $\alpha 3$ gene, it is possible that T_3 -mediated inhibition may involve direct functional interference between $T_3R\beta 1/RXR\beta$ and TATA binding protein. A similar interference mode of action might involve the upstream $T_3R\beta 1/RXR\beta$ -binding site and a recently defined functional CAAT element in this region (35). Alternatively, $T_3R\beta 1/RXR\beta$ heterodimer formation may act directly to repress initiation of $\alpha 3$ gene transcription.

Thyroidal regulation of rat Na,K-ATPase $\alpha 3$ gene expression appears to involve the selective utilization of both stimulatory and inhibitory pathways. For example, a pTRE has

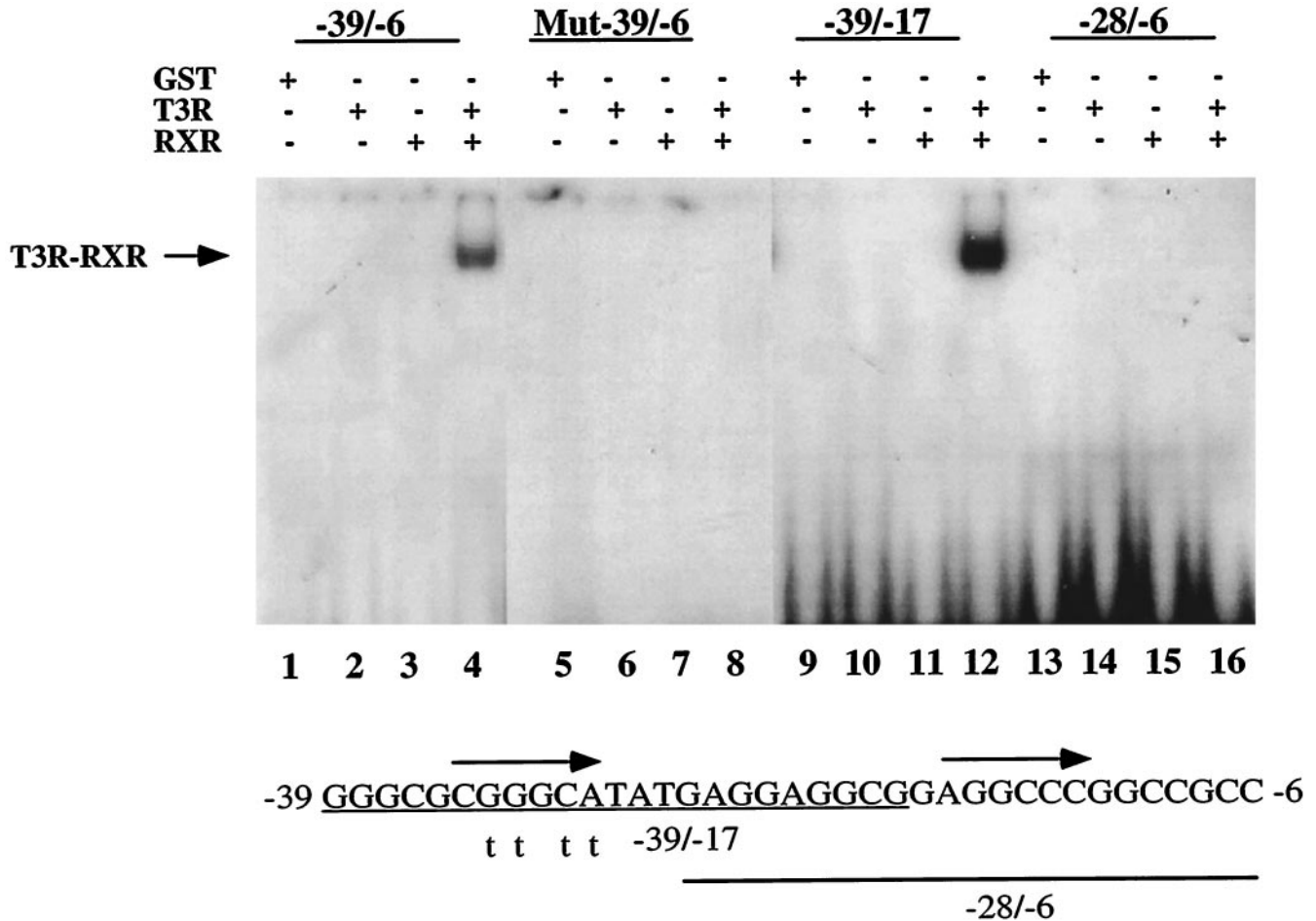


FIG. 9. Localization of $T_3R\beta 1/RXR\beta$ heterodimer binding to nucleotides between -39 and -17 of the $\alpha 3$ gene promoter. ^{32}P -Labeled $\alpha 3$ gene regions $-39/-6$, mutant $-39/-6$, $-39/-17$, and $-28/-6$ were incubated with GST, $T_3R\beta 1$, $RXR\beta$ -GST, or both $T_3R\beta 1$ and $RXR\beta$ -GST as indicated in Fig. 7. Four nucleotides were changed (*lower case*) in the potential T_3R -binding site (*arrow*), which has a four of six match to the AGGTCA motif to generate the mutant $-39/-6$ region (mut $-39/-6$). Reaction samples were resolved by 6% PAGE, and DNA-protein complexes were detected by autoradiography. This result is representative of three experiments.

been proposed to be responsible for the up-regulation of $\alpha 3$ mRNA content observed after a 1-day exposure of primary cultures of neonatal rat cardiac myocytes to T_3 (11). In this study exposure to T_3 for either 2 or 3 days, however, yielded a 50% decrease in $\alpha 3$ mRNA, suggesting that the pTRE was inactivated, and an inhibitory mechanism was functional. We propose that the nTRE present in the -68 to -6 bp region of the $\alpha 3$ promoter plays a fundamental role in this T_3 inhibitory pathway. A similar switch of T_3 responsiveness was evident in our demonstration that T_3 -mediated repression of $\alpha 3$ gene transcription was abolished when neonatal rat cardiac myocytes were cultured at a high cell density. Finally, $\alpha 3$ mRNA content was unresponsive to T_3 in a rat telencephalon organotypic cell culture system (10). Taken together, these findings indicate that T_3 can stimulate, repress, or have no effect on $\alpha 3$ gene expression and suggests that an interplay between positive and negative transcriptional events contributes to this complex response.

Acknowledgments

We thank Drs. D. Moore and G. Gill for providing rat $T_3R\beta 1$ and mutant rat $T_3R\beta 1$ expression vectors, respectively. The authors are also

grateful to Dr. P. Chambon for providing an expression plasmid for mouse $RXR\beta$. We thank Dr. M. Rosenfeld for rat $RXR\beta$ -GST fusion protein and Dr. W. Solomon for the gift of GST. The authors thank Drs. R. Gaynor and R. Tjian for providing AP2 and Sp1 expression vectors, respectively. We also thank Dr. L. Freedman for the gift of a luciferase reporter construct containing a pRXRE.

References

- Jorgensen PL 1982 Mechanism of the Na^+, K^+ pump. *Biochim Biophys Acta* 694:27-68
- MacKnight ADC, Leaf A 1977 Regulation of cellular volume. *Physiol Rev* 57:510-573
- Lee CO 1985 200 years of digitalis: the emerging central role of the sodium ion in the control of cardiac force. *Am J Physiol* 249:C367-C378
- Lingrel JB, Orlovski J, Shull MM, Price EM 1990 Molecular genetics of Na, K -ATPase. *Prog Nucleic Acids Res Mol Biol* 38:37-89
- Lucchesi PA, Sweader KJ 1991 Postnatal changes in Na, K -ATPase isoform expression in rat cardiac ventricle. *J Biol Chem* 266:9327-9331
- Levenson R 1994 Isoforms of Na, K -ATPase: family members in search of function. *Rev Physiol Biochem Pharmacol* 123:1-45
- Ismail-Beigi F, Edelman IS 1970 Mechanisms of thyroidal calorigenesis: role of active sodium transport. *Proc Natl Acad Sci USA* 67:1071-1078
- Gick GG, Ismail-Beigi F, Edelman IS 1988 Thyroidal regulation of rat renal and hepatic Na, K -ATPase gene expression. *J Biol Chem* 263:16610-16618
- Gick GG, Melikian J, Ismail-Beigi F 1990 Thyroidal enhancement of rat myocardial Na, K -ATPase: preferential expression of $\alpha 2$ activity and mRNA abundance. *J Membr Biol* 115:273-282
- Corthesy-Theulaz I, Merillat A-M, Honegger P, Rossier BC 1991 Differential

- regulation of Na,K-ATPase isoform gene expression by T_3 during rat brain development. *Am J Physiol* 261:C124–C131
11. **Kamitani T, Ikeda U, Muto S, Kawakami K, Nagano K, Tsuruya Y, Oguchi A, Yamamoto K, Hara Y, Kojima T, Medford RM, Shimada K** 1992 Regulation of Na,K-ATPase gene expression by thyroid hormone in rat cardiocytes. *Circ Res* 71:1457–1464
 12. **Liu B, Huang F, Gick G** 1993 Regulation of Na,K-ATPase $\beta 1$ mRNA content by thyroid hormone in neonatal rat cardiac myocytes. *Cell Mol Biol Res* 39:221–229
 13. **Huang F, He H, Gick G** 1994 Thyroid hormone regulation of Na,K-ATPase $\alpha 2$ gene expression in cardiac myocytes. *Cell Mol Biol Res* 40:41–52
 14. **He HP, Chin S, Zhuang K, Hartong R, Apriletti J, Gick G** 1996 Negative regulation of the rat Na,K-ATPase $\alpha 3$ subunit gene promoter by thyroid hormone. *Am J Physiol* 271:C1750–C1756
 15. **Brent GA, Larsen PR, Harney JW, Koenig RJ, Moore DD** 1989 Functional characterization of the rat growth hormone promoter elements required for induction by thyroid hormone with and without co-transfected $\beta 1$ type thyroid hormone receptor. *J Biol Chem* 264:178–182
 16. **Glass CK, Holloway JM, Devary OV, Rosenfeld MG** 1988 The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* 54:313–323
 17. **Naar AM, Boutin J-M, Lipkin SM, Yu VC, Holloway JM, Glass CK, Rosenfeld MG** 1991 The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* 65:1267–1279
 18. **Umesono K, Murakami KK, Thompson CC, Evans RM** 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* 65:1255–1266
 19. **Glass CK** 1994 Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr Rev* 15:391–407
 20. **Yu VC, Delsert C, Anderson B, Holloway JM, Devary OV, Naar AM, Kim SY, Boutin J-M, Glass CK, Rosenfeld MG** 1991 RXR β , a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 67:1251–1266
 21. **Bugge TH, Pohl J, Lonnoy O, Stunnenberg HG** 1992 RXR α , a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J* 11:1409–1418
 22. **Tsai M-J, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451–486
 23. **Koenig RJ, Warne RL, Brent GA, Harney JW, Larson PR, Moore DD** 1988 Isolation of a cDNA clone encoding a biologically active thyroid hormone receptor. *Proc Natl Acad Sci USA* 85:5031–5035
 24. **Xu J, Thompson KL, Shephard LB, Hudson LG, Gill GN** 1993 T_3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. *J Biol Chem* 268:16065–16073
 25. **Leid M, Kastner P, Lyons R, Nakashatri H, Saunders M, Zacharewski T, Chen J-Y, Staub A, Garnier J-M, Mader S, Chambon P** 1992 Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68:377–395
 26. **Lemon BD, Freedman LP** 1996 Selective effects of ligands on vitamin D3 receptor- and retinoid X receptor-mediated gene activation *in vivo*. *Mol Cell Biol* 16:1006–1016
 27. **Williams T, Admon A, Luscher B, Tjian R** 1988 Cloning and expression of AP2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev* 2:1557–1569
 28. **Courey AJ, Tjian R** 1988 Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55:887–898
 29. **Ribeiro RCJ, Kushner PJ, Apriletti JW, West BL, Baxter JD** 1992 Thyroid hormone alters *in vitro* DNA binding of monomers and dimers of thyroid hormone receptors. *Mol Endocrinol* 6:1142–1152
 30. **Shull, JD** 1991 Population density alters the responsiveness of GH $_4$ C $_1$ pituitary tumor cells to 17 β -estradiol. *Endocrinology* 129:1644–1652
 31. **Williams PM, Navre M, Ringold GM** 1991 Glucocorticoid induction of the adipocyte clone 5 gene requires high cell density. *Mol Endocrinol* 5:615–618
 32. **Desai-Yajnik V, Samuels HH** 1993 The NF- κ B and Sp1 motifs of the human immunodeficiency virus type 1 long terminal repeat function as novel thyroid hormone response elements. *Mol Cell Biol* 13:5057–5069
 33. **Warshawsky D, Miller L** 1995 Tissue-specific *in vivo* protein-DNA interactions at the promoter region of the *Xenopus* 63 kDa keratin gene during metamorphosis. *Nucleic Acids Res* 23:4502–4509
 34. **Pathak BG, Pugh DG, Lingrel JB** 1990 Characterization of the 5'-flanking region of the human and rat Na,K-ATPase $\alpha 3$ gene. *Genomics* 8:641–647
 35. **Murakami Y, Ikeda U, Shimada K, Kawakami K** 1997 Promoter of the Na,K-ATPase $\alpha 3$ subunit gene is composed of *cis* elements to which NF-Y and Sp1/Sp3 bind in rat cardiocytes. *Biochim Biophys Acta* 1352:311–324
 36. **Carr FE, Wong NCW** 1994 Characterization of a negative thyroid hormone response element. *J Biol Chem* 269:4175–4179
 37. **Sanchez-Pacheco A, Palomino, T, Aranda A** 1995 Negative regulation of expression of the pituitary-specific transcription factor GHF-1/Pit-1 by thyroid hormones through interference with promoter enhancer elements. *Mol Cell Biol* 15:6322–6330
 38. **Hollenberg AN, Monden T, Flynn TR, Boers M-E, Cohen O, Wondisford FE** 1995 The human thyrotropin-releasing hormone gene is regulated by thyroid hormone through two distinct classes of negative thyroid hormone response elements. *Mol Endocrinol* 9:540–550
 39. **Cohen O, Flynn TR, Wondisford FE** 1995 Ligand-dependent antagonism by retinoid X receptors of inhibitory thyroid hormone response elements. *J Biol Chem* 270:13899–13905
 40. **Haugen BR, Brown NS, Wood WM, Gordon DF, Ridgway EC** 1997 The thyrotrope-restricted isoform of the retinoid-X receptor- $\gamma 1$ mediates 9-*cis*-retinoic acid suppression of thyrotropin- β promoter activity. *Mol Endocrinol* 11:481–489
 41. **Pennathur S, Madison LD, Kay TWH, Jameson, JL** 1993 Localization of promoter sequences required for thyrotropin-releasing hormone and thyroid hormone responsiveness of the glycoprotein hormone α -gene in primary cultures of rat pituitary cells. *Mol Endocrinol* 7:797–805
 42. **Tzamelis I, Zannis VI** 1996 Binding specificity and modulation of the ApoA-I promoter activity by homo- and heterodimers of nuclear receptors. *J Biol Chem* 271:8402–8415
 43. **Rahman A, Esmaili A, Saatcioglu F** 1995 A unique thyroid hormone response element in the human immunodeficiency virus type 1 long terminal repeat that overlaps the Sp1 binding sites. *J Biol Chem* 270:31059–31064