Identification of a functional thyroid hormone response element in the upstream flanking region of the human Na,K-ATPase β 1 gene

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ABSTRACT

The human Na,K-ATPase β 1 subunit gene promoter activity is stimulated by thyroid hormone (T3) in the human intestinal Caco-2 cells. To identify potential *cis*-acting transcriptional regulatory elements involved in this process, chimeric plasmids containing varying lengths of the 5' flanking region of the human β 1 Na,K-ATPase gene linked to the firefly luciferase reporter gene were introduced into Caco-2 cells by transient transfection. Analysis of T3-regulated luciferase activity of cells carrying these plasmids, and subsequent use of site-directed mutagenesis revealed that a region from –459 to –438 (relative to the transcriptional start site) is required for the induction of the β 1 Na,K-ATPase gene by T3. An oligonucleotide containing this sequence from –465 to –433 confers T3 responsiveness to a heterologous promoter. Gel mobility shift assays showed specific binding of nuclear proteins of Caco-2 cells to this region and immunoreactive T3 receptor was identified in one of these complexes. These data demonstrate that there is a *cis*-acting thyroid hormone responsive element in the 5' flanking region of the human β 1 Na,K-ATPase gene and induction of transcription of this gene by T3 involves specific binding of the thyroid hormone receptor to the TRE located at position –459 to –438.

INTRODUCTION

The Na,K-ATPase is a ubiquitous transmembrane protein found in cells of higher organisms and transports Na⁺ and K⁺ ions across the plasma membrane in an ATP-dependent manner. The enzyme is composed of an α subunit and a smaller glycosylated β subunit in equal molar ratios. The α subunit mediates the catalytic activity of the enzyme and contains the specific binding site for cardiac glycosides. While the exact role of the β subunit in enzyme function is unclear, its presence is required for functional enzyme activity as demonstrated by coexpression studies in both *Xenopus* oocytes and in yeast (1,2). It has been

proposed that the β subunit may facilitate the correct assembly and transport of the α subunit from the endoplasmic reticulum to the plasma membrane (3,4) and contribute to the protein folding and stability (5,6). Recent studies have established that Na,K-ATPase belongs to a multigene family. Three distinct isoforms of the α subunit (α 1, α 2 and α 3) and the β subunit (β 1, β 2 and β 3) have been described (7–12). The α and β isoform genes are expressed in a tissue and cell-specific manner and are subject to developmental (13–16) and hormonal (17) regulatory influences.

It is well established that thyroid hormone (T3) stimulates Na,K-ATPase activity in a wide variety of tissues including kidney, intestine, skeletal muscle and cardiac muscle (18–21). The changes in enzyme activity are concomitant with changes in the α and β isoform mRNAs and protein levels (22–24). T3 regulation of Na,K-ATPase gene expression involves a complex interaction of transcription, post-transcriptional, translational, and post-translational mechanisms. A combination of these processes appears to be utilized in a tissue-specific manner. T3 augments both the activity and the relative abundance of different isoforms of rat brain Na,K-ATPase during development (25). This regulation of the gene expression by T3 in rat fetal brain is largely mediated at the post-transcriptional level (25). In contrast, in rat kidney cortex, T3 augments α 1 and β 1 Na,K-ATPase gene expression by increasing their rate of gene transcription, although post-transcriptional processes may also contribute to the overall regulation (26). This implies that Na,K-ATPase genes may contain T3-responsive DNA regulatory elements. It has not been established whether the primary effect of T3 induction of transcription involves direct interaction of a nuclear T3-receptor complex with *cis*-acting regulatory elements of α 1 and β 1 genes or whether the induction is secondary to T3 induction of other regulatory proteins that mediated enhanced transcription of the Na,K-ATPase genes.

Studies of T3 regulation of other genes have demonstrated that the increase in transcription is a result of the direct interaction of the T3 receptor with a TRE (27–29). Brent et al. (30) have proposed that the binding of the T3-receptor requires two half-

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site hexamers, AGGT(C/A)A, for the T3 response. The limited TREs identified thus far, however, show a greater flexibility in half-site arrangement and orientation. At present, functional TREs for the α and β isoform of Na,K-ATPase genes have yet to be identified.

Previous studies from our laboratory have shown that a human β 1 Na,K-ATPase chimeric gene is T3-responsive in human intestinal epithelial cells (Caco-2) (31). Therefore these cells may be a useful model for studying thyroid hormone regulation of Na,K-ATPase gene expression. We have focused on examining the essential role of the transcriptional process in mediating T3 regulation of the β 1 Na,K-ATPase gene. The result indicated that only the TRE at -455 is functional in the human β 1 Na,K-ATPase gene. Examination of the 5'-flanking region of the human β 1 Na,K-ATPase gene reveals three potential thyroid hormone response elements (TREs) at positions -455, -226 and -99 relative to the transcriptional start site (32).

MATERIALS AND METHODS

Plasmid construction

Plasmids containing the human β 1 Na,K-ATPase gene promoter and various lengths of 5' flanking region fused to a promoterless luciferase gene were constructed in the pOLUC reporter plasmid (33). Fragments of the human β 1 Na,K-ATPase gene from 5' SalI (-1141), XbaI (-726), XhoI (-327) and NaeI (-82) to a PvuII site +491 bp from the transcription start site (the translation start site of the β 1 subunit gene is located at +511) were isolated and ligated into SalI and/or SmaI sites in the polylinker of pOLUC 5' of the luciferase gene. These constructs are named pH β 1-1141LUC, pH β 1-726LUC, pH β 1-327LUC and pH β 1-82LUC. Subsequent deletion plasmids pH β 1-554LUC, pH β 1-429LUC and pH β 1-119LUC were made by exonuclease III digestion in the modified pH β 1-1141LUC plasmid where unique restriction sites KpnI and MluI were created 5' of the β 1 gene by insertion of an adaptor with two cohesive ends.

The BamHI fragments from -554 to -296 released from plasmid pH β 1-554LUC were inserted into the BamHI site in the polylinker of pH β 1-119LUC and pH β 1-82LUC. These two constructs are referred to as pH β 1-554 Δ (-295/-120)LUC, pH β 1-554 Δ (-295/-83)LUC. Plasmids pH β 1-554 Δ 1LUC, pH β 1-554 Δ 2LUC, pH β 1-554 Δ 3LUC with 5' up to -554 containing a deletion at -483 to -463, -459 to -449, and -448 to -438 respectively were made by site-directed mutagenesis (34). A BamHI cassette from the wild type human β 1 gene (-554 to -296) was cloned into M13 mp19 to make mutations. Mutations were verified by DNA sequencing. Subsequently, the BamHI cassette was released by restriction enzyme digestion and ligated with the plasmid containing the rest of the wild type β 1 gene fused to the luciferase gene.

A plasmid TKLUC was constructed with the HSV thymidine kinase promoter (-105 to +51) (35) ligated into BamHI-SmaI sites of pOLUC. Double stranded synthetic oligonucleotides β 1TRE and β 1MutTRE containing either wild type sequence of the β 1 gene from -465 to -433 or with substitutions at bases -455, -451, -450, -442, -441, C to A and -443, A to T were inserted into SalI-BamHI sites upstream of the thymidine kinase promoter in pTKLUC. These plasmids are named pH β 1TRETKLUC and pH β 1MutTRETKLUC. The oligonucleotides were synthesized in such a manner so that they contained a SalI site at the 5' end and a BamHI site at the 3'

end. The sequence (sense strand) of the oligonucleotides are shown as follows: β 1TRE (-465 to -433) 5' TCGACGCC-AGTCCGGCGTCCCGGAGTGACCTTCCCCCG 3'; β 1MutTRE 5' TCGACGC CAGTCCGGAGTAACGGAGTG TAATTCCCCCG 3' (substitutions are underlined). Both the sense and anti-sense strands were synthesized separately and annealed to generate a double stranded product which was sequentially cloned into the appropriate restriction sites as described above. The structure of all plasmids was confirmed by restriction mapping and/or sequencing.

Cell culture

Caco-2, a human intestinal cell line, was maintained in Dulbecco's modified Eagle's high glucose medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO), penicillin 100 U/ml and streptomycin 100 μ g/ml. The cells were grown for 7 seven days and passaged by trypsinization in the presence of EDTA, followed by replating at a dilution 1:5. The cells were plated overnight and used for hormone studies after a 24 hour incubation in fresh medium supplemented with fetal bovine serum pretreated with AG1-X8 resin and activated charcoal to eliminate thyroid and steroid hormones (called 'stripped' FBS). The cells were then used for T3 induction of the endogenous β 1 gene and for transient transfection assays of the exogenous chimeric β 1 genes.

RNA isolation and Northern analysis

The cells were harvested after 24-72 hours treatment of 50 nM T3 and RNA was prepared for Northern RNA blot analysis. Total RNA was isolated from control cell cultures and from those T3 treated cell cultures according to the method of Chomczynski and Sacchi (36). RNA concentration was determined spectrophotometrically. Total cellular RNA samples (10 μ g/each) were denatured with glyoxal and dimethylsulfoxide (DMSO), size-fractionated by electrophoresis on 1.0% agarose gels and transferred to Nytran nylon membranes (MSI) in 10 $\times$ SSC. The membranes were baked, prehybridized and hybridized by using the method described by Church and Gilbert (37). The hybridization probe was a HindIII/PstI fragment from the rat β 1 cDNA corresponding to nucleotides 25 to 296 that was labelled with α -³²P-dCTP by random oligonucleotide priming. A 20 mer synthetic oligonucleotide based on the human 18S ribosomal RNA (GACAAGCATATGCTACTGGC, ref. 38) was used as a control for the quantity of RNA bound to the membrane. The end-labeled oligonucleotide with γ -³²P-ATP was used as a probe and a 30 fold molar excess of the unlabeled probe (relative to the total RNA in the blot) was also added in the hybridization to obtain signals in the linear range. The blots were exposed to a Phosphorimage Analyzer screen (Molecular Dynamics) to quantitate the radioactive signals.

Transfection and assays of the transfected cell extracts

Following plating overnight, the cells were washed twice with PBS and changed to DMEM supplemented with 'stripped' fetal bovine serum at least two hours before transfection. DNA transfection of Caco-2 cells was carried out by calcium phosphate precipitation (39) with 20 μ g of pH β 1LUC plasmid DNA and 4 μ g of RSV110 (β -galactosidase expression plasmid). After a 20 hour precipitation period at 3% CO₂, 35°C, the cells were washed twice and changed to fresh 'stripped' media with or without 50nM T3 for 48 hours. The cells were lysed in 500 μ l triton glycylglycine lysis buffer. Cell extracts were diluted in 25

mM glycylglycine (pH 7.8) and assayed for luciferase activity as previously described (40). β -galactosidase activity served as an internal control to monitor for transfection efficiency. Detection of β -galactosidase activity was performed according to a standard protocol obtained from Pharmacia (Analects 14). Six separate transfections of two preparations of each plasmid were performed.

Preparation of nuclear extracts

Nuclear extracts were prepared from Caco-2 cells according to the procedure described by Digman et al (41) except that all buffers contained leupeptin (0.5 μ g/ml), pepstatin (1 μ g/ml) and phenylmethylsulfonyl fluoride (PMSF, 0.5 mM). The final extracts subsequently dialyzed for 4 hours against 50 volumes of a buffer containing 20% glycerol, 20 mM Hepes pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT) and three proteinase inhibitors as above. The protein concentrations were determined according to the method of Bradford (42).

Probes, competitors and gel mobility shift assays

Different double stranded oligonucleotides were radiolabeled for use as probes or added unlabeled as competitors. Oligonucleotides were prepared on an Applied Biosystems DNA synthesizer and their sequences (antisense strand except that rGHTRE and γ -globin site I are sense strand) are shown as follows (the other strand is complemented).

β 1TRE	5' GATCCGGGGGAAGGTCCTCCGGGACGCC-GGACTGGCG3'
β 1MutTRE	5' GATCCGGGGGAATTACACTCCGTTACTCC-GGACTGGCG3'
β 1-226	5' GCGGGGGCGGCGGTGAGGGACGCGCAGG-CAGC3'
β 1-99	5' CCAGGGGCGAGGGACTCGGCCCGAGAGGG-CGGGTT3'
rGHTRE	5' AAAGGTAAAGATCAGGGACGTGACCGC3'
α MHCTRE	5' TGGAGGTGACAGGAGGACAGCAGCCCT3'
γ -globin site I	5' GATCGGAAGAATAAATTAGACTAAAAAC-TGGA3'

β 1TRE corresponds to a partial sequence of the human Na,K-ATPase β 1 gene promoter (−433 to −465) containing the functional TRE. β 1MutTRE contains designed mutations indicated by bold letters to disrupt two half-sites present in the functional TRE of the β 1 gene. β 1-226 and β 1-99 contain the partial sequences of the β 1 Na,K-ATPase gene from −206 to −238 or −87 to −118 that are homologous to TRE consensus site. rGHTRE and α MHCTRE represent the TREs in the rat growth hormone gene (−190 to −167) or in the human α myosin heavy chain gene (−133 to −158). γ -globin site I used as a nonspecific competitor was a 31 bp oligonucleotide from the human γ -globin promoter (−293 to −267). The oligonucleotides with single-stranded tails on both ends were annealed, purified by 12% PAGE (1×TBE) and end-labeled using T4 polynucleotide kinase and γ - 32 P-ATP.

5–10 μ g of nuclear extracts total protein and 50,000 cpm oligonucleotides probes (0.1–0.2 ng) were incubated in 20 μ l of buffer contained 30 nM T3, 10 mM Hepes (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 0.2 mM β -mercaptoethanol, 0.1% NP40, 1 μ g/ μ l BSA, 0.2 μ g/ μ l of double stranded poly(dA-dT) heteropolymer and 4% Ficoll for 30 min at room temperature. Specific double stranded oligonucleotides were included as competitors. After incubation, samples were subjected to electrophoresis on 5% polyacrylamide gels in a 0.25× TBE buffer.

For immunoassays, after incubation of binding reaction at room temperature, polyclonal antibody (1:60) against rat T3 receptor β 1 isoform amino acid 73–93 (43) (human T3 receptor β has the same epitope) or preimmune serum was added and incubated for another 30 min at room temperature, then subjected to electrophoresis on 4% polyacrylamide gel in 0.25× TBE buffer. The gels were dried and autoradiographed with an intensifying screen. Film exposure to the gels ranged from 8h to 24h.

RESULTS

T3 induction of expression of the endogenous β 1 gene expression in Caco-2 cells

To quantitatively measure endogenous Na,K-ATPase β 1 gene expression in Caco-2 cells in response to T3, total cellular RNA was isolated from cells exposed to 50 nM T3 for 24–72 hours and subjected to Northern blot analysis. The β 1 subunit probe hybridized to two RNA species of 2.7 and 2.35 kb (Fig. 1). The abundance of β 1 mRNA was increased after treatment with T3 compared to the control samples. Quantitation of these changes was obtained by Phosphorimager analysis revealing a 3 fold induction following 24 or 48 hours of T3 treatment which then decreased after 72 hours of treatment. The amount of RNA present in each lane was normalized for minor loading differences relative to 18S RNA.

Transient transfection of 5' deletion constructs in Caco-2 cells

To analyze T3 regulation of human Na,K-ATPase β 1 promoter activity, plasmid pH β 1-1141LUC containing 1632bp (−1141 to +491) of the human β 1 promoter was inserted upstream of the luciferase reporter gene. This construct was transfected into Caco-2 cells and was assayed for luciferase activity following incubation of the cells for 48 hours in the absence or presence 50 nM T3. Luciferase activity increased 4.1 fold following T3 addition. Examination of this sequence revealed three potential TREs, Fig. 2A. To determine if one or all of the TREs were functional, plasmids containing the luciferase gene attached to

Figure 1. Induction of Na,K-ATPase β 1 subunit mRNA by thyroid hormone in Caco-2 cells. Caco-2 cells were cultured for 24–72 hours in the presence or absence of 50nM T3. Total cellular RNA was isolated and examined in a Northern RNA analysis. The radiolabeled hybridization probe was a HindIII/PstI fragment of the rat β 1 subunit cDNA. γ - 32 P-ATP end-labeled 18S oligonucleotide was used to hybridize the same blot to demonstrate the loading in each lane. – is total RNA from cells grown in 'stripped' medium; + is total RNA from cells grown in 'stripped' medium plus 50 nM T3.

Figure 2. A, Sequence of the human Na,K-ATPase $\beta 1$ subunit gene 5'-flanking region. The sequence is numbered relative to the transcriptional start site at position +1. Consensus sequences for CAAT and TATA boxes are indicated by bold letters. Three potential TREs underlined contain consensus binding sites for T3 receptor on the antisense strand. **B**, T3 induction of luciferase expression of various 5' deletion constructs in Caco-2 cells. Induction levels are expressed as the ratio of luciferase activities in T3-treated versus untreated cells. All data are averages $\pm$ SD of six separate transfection experiments performed in duplicates. Three potential TREs are indicated by bars labeled with the nucleotide positions. The plasmids are named based on the endpoints of the $\beta 1$ subunit gene region in each 5' deletion luciferase construct.

varying lengths of the 5' flanking region of the $\beta 1$ promoter were transfected into Caco-2 cells and tested for their T3 response. Fig. 2B shows the 5' deletion constructs and the level of T3 induction obtained for each construct. Plasmids with endpoints at positions -726 and -554 were responsive to T3 (2.8 and 4.7 fold respectively). However, deletion of an additional 125 bp from position -554 to -429 completely abolished T3 inducibility (stimulation dropped from 4.7 to 0.8 fold). Further deletions in the 5' flanking region with endpoints at positions -327, -119, and -82 did not confer T3 inducibility despite the fact that two potential TREs at positions -226 and -99 are present in pH $\beta 1$ -327LUC. The observed lack of induction of these plasmids is not due to insufficient basal gene expression. All the constructs retained their basal activity. These results demonstrate

Figure 3. T3 induction of luciferase expression of the H $\beta 1$ -554 internal deletion constructs. Construct H $\beta 1$ -554 Δ (-295/-120) deleted sequences including one downstream putative TRE at -226. H $\beta 1$ -554 Δ (-295/-83) deleted sequences including two downstream putative TREs at -226 and -99. Induction levels are expressed as the ratio of luciferase activities in T3-treated versus untreated cells. All data are averages $\pm$ SD of six separate transfections.

Figure 4. Effect of deletions in the consensus motifs of TRE on T3 induction of the $\beta 1$ subunit gene. Induction levels are expressed as the ratio of luciferase activities in T3 treated versus untreated cells. All data are averages $\pm$ SD of six separate transfections. Site-directed mutagenesis in the consensus TRE motifs deletes 11 bp at -459 to -449 and -448 to -438 and a 21 bp at -483 to -463 5' of the consensus TRE motifs. These constructs are referred to as pH $\beta 1$ -554 Δ 2LUC, pH $\beta 1$ -554 Δ 3LUC and pH $\beta 1$ -554 Δ 1LUC, respectively.

Figure 5. T3 induction of luciferase expression from TRE of the $\beta 1$ gene and its mutant linked to TKLUC. Induction levels are expressed as the ratio of luciferase activities in T3-treated versus untreated cells. All data are averages $\pm$ SD of six separate transfections.

that the thyroid hormone response element of the $\beta 1$ gene of Na,K-ATPase is located within nucleotides -554 to -429, a region that contains one of the putative TREs at position -455.

To determine whether the other two putative TREs at -226 and -99 are required for the T3 responsiveness, two additional constructs were made [pH $\beta 1$ -554 Δ (-295/-120) and pH $\beta 1$ -554 Δ (-295/-83)] that contained internal deletions of one or both of the putative TREs and these were tested for their ability to confer T3 responsiveness. The results of these assays are presented in Fig. 3 and reveal that both deletions retain full T3 inducibility. Thus, these two downstream putative TREs are

Figure 6. Gel mobility shift analysis of the β 1 TRE with nuclear extracts from Caco-2 cells. **A**, Competition analysis of the specific binding activities of the β 1TRE. The γ - 32 P-labeled oligonucleotide β 1TRE was incubated with nuclear extracts prepared from Caco-2 cells. A, B, C and D denotes protein-DNA complexes identified according to their mobility relative to the origin. F indicates free probe or unbound radiolabeled probe. * is nonspecific binding activity. Different unlabeled oligonucleotides used as competitors at different fold molar excess are indicated on the top of the autoradiograph. **B**, Gel mobility shift/immunoassays. Effect of a TR β -1 directed antiserum on the migration of protein complexes to the β 1TRE. Lane 1: preimmune serum (1:60), lane 2: no serum, and lane 3: antiserum (1:60) was added to each reaction after incubation of nuclear extracts with probe. The position of protein-DNA-antibody complex E is indicated. **C**, Gel mobility shift assays to demonstrate the difference and similarity of binding activities of the three putative TREs in the β 1 subunit gene and one mutant of the functional TRE. Probes used in each lane are indicated on the top of autoradiograph. Unlabeled probes as their specific competitors were added (+ lanes) in 100 $\times$ molar excess amount relative to the probes. The control lanes (- lanes) have no unlabeled competitors. The free probe was run off the gel to obtain better resolution of the complexes.

neither functional nor required for the T3 responsiveness of the β 1 gene.

Site-directed mutagenesis in the consensus motifs of the TRE in the β 1 Na,K-ATPase gene

To more precisely locate the *cis*-acting element of the β 1 Na,K-ATPase gene required for T3 responsiveness, a set of small internal deletions were made by site-directed mutagenesis in the consensus TRE region around position -455. The sequences deleted in three plasmids are shown in Fig. 4. The 11 bp deletions were constructed to separately eliminate each half-site of the possible TRE. As a control, a 21 bp deletion upstream of the putative TRE was also prepared. The two 11 bp deletions at -459 to -449 and at -448 to -438 result in the loss of the T3 response (plasmid constructs pH β 1 -554 Δ 2LUC and pH β 1 -554 Δ 3LUC, respectively). Another 21 bp deletion at -483 to -463 (pH β 1 -554 Δ 1LUC) retained the T3 response (about 3.7 fold). These studies identify the sequence required for T3 induction of the human β 1 Na,K-ATPase gene promoter and locate this TRE at position -459 to -438 relative to the transcription start site.

T3 induction with the putative β 1 TRE and its mutant upstream of the thymidine kinase promoter

To determine if the 22 bp region extending from position -459 to -438 (relative to the transcriptional start site) constituted a

functional T3 regulatory element, a double-stranded synthetic oligonucleotide (β 1TRE) was prepared containing this sequence as well as six or five bp flanking the 5' and 3' end respectively (corresponding to nucleotides -465 to -433 of the β 1 gene). A single copy of this oligonucleotide was inserted in its native orientation upstream of the thymidine kinase (TK) promoter (construct pH β 1TRE TKLUC). Luciferase gene expression was stimulated 2.8 fold by T3 in cells transfected with this construct (Fig. 5). No induction was observed when the oligonucleotide (β 1MutTRE) containing substitutions in the consensus motifs of TRE in the β 1 gene fused to TK promoter. These results demonstrated that the sequence from -465 to -433 of the β 1 gene contains a potential TRE that was sufficient to confer T3 responsiveness.

In vitro DNA binding studies

To determine if thyroid hormone receptor(s) bind this β 1 subunit gene thyroid hormone responsive element (located at -459 to -438 relative to the transcription start site), the double stranded synthetic oligonucleotide β 1 TRE (-465 to -433) was used as a probe to perform gel mobility shift assays. The results (Fig. 6A) show that when this labeled oligonucleotide was incubated with nuclear extracts from Caco-2 cells grown in the stripped media (devoid of thyroid hormone), four retarded bands occur (A, B, C and D) and three of these A, B, and C are effectively competed by the specific competitor. Complex C appears to be competed

Human NKA β 1	-438/-459	<u>AAGG</u> TC <u>ACTCCGGG</u> ACGCGGA
Rat α MHC	-133/-151	<u>AAGG</u> TC <u>GACGAGG</u> AGC
Rat GH	-190/-166	<u>AAGG</u> TA <u>GATCAGG</u> ACGTGACCGC
Rat TSH α	-69/-56	TTAGGTGCAGGTG
Rat Malic Enzyme	-280/-261	TTGGGGTTAGGGGAGGACGT
MoMLV	351/331	CAGGGTCATTTCAGGTCTTG
Human α MHC	-138/-158	GGAGGTGACAGGAGACA
Human hCS-B	-65/-44	GGTGGGGTCAAGCAGGGAGAGA
consensus binding half-site of Brent et al.		AGGTCA A
consensus binding half-site of Glass et al.		GGGTCA A AGG

Figure 7. Comparison of DNA sequences between the human Na,K-ATPase β 1 subunit gene TRE and other reported TREs. Arrows highlight sequences which correspond to a proposed TRE core binding motif and indicate the direction of this motif. Sequences illustrated are from the human Na,K-ATPase β 1 subunit gene -438 to -459 (this work), the rat myosin heavy chain β subunit gene -133 to -151 (45), the rat growth hormone gene -190 to -166 (46), the rat α -TSH gene -69 to -56 (47), the rat malic enzyme gene to -280 to -261 (29), the moloney murine leukemia virus 351 to 331 (44), the human myosin heavy chain β subunit gene -138 to -158 (29), the human placental lactogen B gene -65 to -44 (48), consensus T3 receptor binding half-site of Brent et al. (30), and consensus T3 receptor binding site of Glass et al. (50).

at a lower concentration than the others. Complex D is not competed effectively in this gel but these results are variable as competition is observed in other experiments. None of the shifted species was affected when competition was performed with a 100 fold molar excess of an unrelated oligonucleotide (γ -globin site I) (Fig. 6A, lane 7). When the nuclear extract was made from T3 induced Caco-2 cells, the same pattern of shifts occurred except that the shifted species were less intense with the same amount of protein (data not shown). When the binding assays were performed in the absence of T3, neither the pattern nor migration of shifted species was affected (data not shown). Competition experiments using two previously well defined TREs present in the rat growth hormone (44) and human β myosin heavy chain gene promoters (29) as competitors (100 fold molar excess) showed specific competition of species A and C, (Fig. 6A) indicating that T3 receptor is part of the complexes interacting with the TRE of the β 1 Na,K-ATPase gene. To confirm if these two binding activities directly involved the T3 receptor, an antiserum raised against T3 receptor β -1 (43) was used in the gel shift-immunoassays. The resulting complexes had similar electrophoretic mobility in presence or absence of preimmune serum. In contrast, addition of immune serum resulted in a reduction in the intensity of the electrophoretic shift band C as well as the visualization of a new supershifted species E (Fig. 6B). At the similar conditions, no shifted bands occurred when antiserum alone incubated with the probe (data not shown). This indicates that a T3 receptor or an antigenically closely related protein is a part of complex C. The other bands were not immunoreactive indicating that either the epitope in the complex cannot be detected or they are not related to T3 receptor. When the functional TRE of the β 1 subunit gene was mutated (β 1MutTRE) and used in the gel shift analysis, no complex A

and C were observed (Fig. 6C, lane 5,6) although enhanced binding of complex D was found (Fig. 6C).

The two putative TREs in the β 1 subunit gene positioned at -226 (β 1-226) and -99 (β 1-99) that were not functional were also tested for their DNA-protein binding activity. Although the same migration species A, B and D were observed with varied intensity, they did not promote the formation of complex C, the complex that is shown above to contain the T3 receptor (Fig. 6C, compare lanes 1 and 2, 7 and 8 with the control 3 and 4). These results individually and collectively indicate that T3-induced transcription of the β 1 subunit of the Na,K-ATPase gene functions through a TRE that interacts directly with the T3 receptor.

DISCUSSION

It is well established that the α and β isoform subunits of the Na,K-ATPase gene family are differentially regulated by thyroid hormone in a tissue- and cell-specific manner. More recent studies have begun to focus on the molecular mechanisms by which T3 influences Na,K-ATPase gene expression. These initial studies appear to indicate that both transcriptional and post-transcriptional events contribute to this process, although the precise molecular events remain to be elucidated. We have recently reported that the Na,K-ATPase α 1 and β 1 mRNAs and corresponding α 1/ β 1 enzyme activities are positively stimulated by T3 in the human intestinal Caco-2 cell line (31). The activation of the β 1 subunit chimeric gene was further shown to be mediated by a transcriptional process. We now extend these results by locating the minimal DNA sequence element of the β 1 subunit gene that is required for transactivation by T3. Using 5' deletion and site-directed mutagenesis of regions in the 5'-flanking region of the β 1 subunit gene linked to the luciferase reporter gene, we show that the sequence spans 22 bp from -459 to -438. The oligonucleotide containing this sequence is sufficient to transfer T3 inducibility to the heterologous HSVTK promoter. The anti-sense strand of this sequence contains two hexamers (5' GAAGGTCACTCCGGGACGCGGA 3') that agree with the consensus half-binding site [AGGT(C/A)A] of the T3 receptor (30,44). One of these half-sites is an exact consensus match, while the other exhibits only a 3/6 match. However, when either of these two motifs are deleted in the β 1 subunit gene, T3 responsiveness is lost. Interestingly, the other two downstream putative TREs (positions -226 and -99) that have at least one copy of the consensus half-site are not functional. Deletions of these latter two sequences did not affect T3 induction of the β 1 subunit gene.

Several TREs have been identified by transfection with deletion mutants and/or by in vitro binding analysis (see summary Fig. 7). These TREs usually contain two purine-rich motifs that closely resemble, but are not identical to, the proposed consensus T3 receptor binding site. Similar to steroid receptor consensus sequences, a thyroid hormone response element appears to require at least two copies of this half-site to generate a functional T3 response element (30). However, unlike steroid hormone response elements, the arrangement and orientation of the half-sites can tolerate greater variation and still maintain function. Regarding the human Na,K-ATPase β 1 subunit gene, the TRE resembles an imperfect, direct repeat that conforms to the half-site consensus AGGT(C/A)A motif for receptors bound to thyroid hormone, retinoic acid, α -cis retinoic acid, vitamin D3 and estrogen. Furthermore, the tandem TREs are separated by a four-nucleotide spacing that has recently been implicated as an

important component of the TRE (51). Studies by Umesono et al. (51) have shown that the nucleotide spacing between the direct repeats of hormone response elements plays an important physiological role in achieving hormone specificity. Accordingly, nucleotide spacing of 3, 4 and 5 nucleotides between the two half-sites specifies a selective response to vitamin D3, T3 and retinoic acid receptors, respectively.

Analysis of the DNA-binding properties of the T3 receptor has shown that T3 receptors can bind as monomer, dimer and TR α /TR β dimer (52). Additionally, TRs heterodimerize with nuclear proteins such as T3 auxiliary protein (TRAP), retinoic acid receptor (RAR), and retinoid X receptors (RXR) (53–57). These heterodimers appear to bind better to TREs than homodimers and may play a role in T3-regulated transcription (56,57). To examine the mechanism of T3 regulation of transcription of the β 1 subunit gene, we performed a series of gel mobility shift assays. Using crude nuclear extracts of Caco-2 cells, two prominent species (A and C) and two less prominent species (B and D) can be shifted by the oligonucleotide of the β 1 subunit gene sequence (–465 to –433) that transfer the T3 induction to the TK promoter. The same pattern was obtained using nuclear extracts from cells grown either in 'stripped' media or in T3 induced media. It seems that no new binding activities to this site occur after T3 treatment by this assay and implies that T3 is not essential for binding, at least in vitro. It has been demonstrated that the thyroid hormone receptor can bind to its specific DNA response element in vitro in the absence or presence of T3 (58). However, transcriptional activation depends on the presence of T3. The species C is a unique retarded band found only with the oligonucleotide containing the functional TRE and it is supershifted by a specific anti-TR β -1 antibody indicating T3 receptor or an antigenically related protein is part of the complex. Similar results were observed with anti-rat T3 receptor polyclonal antibody (gift of Dr Tuymirat) (data not shown). We noticed that increased abundance of the supershift species E was not quantitatively associated with an equivalent decrease of species C. This is possible because antibodies may destabilize the DNA-T3 receptor interactions or most of the DNA–protein–antibody complex precipitates out of solution. Although species A was not immunoreactive to T3 receptor antibody, it was competed by two well-characterized TREs from the rGH and human α MHC genes suggesting that species A contains a factor(s) involving interaction with a TRE either unrelated to T3 receptor or forming a T3 receptor complex such that the epitope cannot be detected by the antibody because of steric hindrance. Furthermore, substitutions in the consensus sites abolished both species A and C completely in the in vitro binding assays and also lost T3 induction of the heterologous promoter. Based on these results and functional data, we conclude that T3 induction of the human Na,K-ATPase β 1 subunit gene transcription involves a direct T3 receptor–DNA interaction. However, our results do not preclude the possibility that other nuclear protein(s) (for example, species A) may also play a role.

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