Identification of a Thyroid Hormone Response Element in the Mouse Krüppel-Like Factor 9 Gene to Explain Its Postnatal Expression in the Brain

Robert J. Denver and Keith E. Williamson

Department of Molecular, Cellular, and Developmental Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048

Brain development is critically dependent on thyroid hormone (T3). Krüppel-like factor 9 (Klf9) is a T3-inducible gene in developing rat brain, and several lines of evidence support that KLF9 plays a key role in neuronal morphogenesis. Here we extend our findings to the mouse and demonstrate the presence of a functional T3 response element (T3RE) in the 5' flanking region of the mouse Klf9 gene. Klf9 mRNA is strongly induced in the mouse hippocampus and cerebellum in a developmental stage- and T3-dependent manner. Computer analysis identified a near optimal direct repeat 4 (DR-4) T3RE 3.8 kb upstream of the Klf9 transcription start site, and EMSAs showed that T3 receptor (TR)-retinoid X receptor heterodimers bound to the T3RE with high affinity. The T3RE acts as a strong positive response element in transfection assays using a minimal heterologous promoter. In the mouse neuroblastoma cell line N2a[TR/H11032], T3 caused a dose-dependent up-regulation of Klf9 mRNA. Chromatin immunoprecipitation assays conducted with N2a[TR/H11032] cells showed that TRs associated with the Klf9 T3RE, and this association was promoted by T3. Treatment of N2a[TR/H11032] cells with T3 led to hyperacetylation of histones 3 and 4 at the T3RE site. Furthermore, TRs associated with the DR-4 T3RE in postnatal d 4 mouse brain, and histone 4 acetylation was greater at this site compared with other regions of the Klf9 gene. Our study identifies a functional DR-4 T3RE located in the mouse Klf9 gene to explain its regulation by T3 during mammalian brain development.

(Endocrinology 150: 3935–3943, 2009)
In the present study, we investigated the molecular basis for T₃ regulation of the Klf9 gene in developing mouse brain. We first confirmed that Klf9 is developmentally regulated and induced by T₃ in the mouse brain as we earlier found it to be in the rat (13). We next analyzed the mouse Klf9 gene and its 5' flanking region for the presence of putative T₃REs. We identified a near optimal DR-4 T₃RE located about 3.8 kb upstream of the transcription start site. Using EMSA, in vitro transfection assay and chromatin immunoprecipitation (ChIP) assay, we tested whether this DR-4 T₃RE was functional in both cell culture using N2a[TRβ1] cells and neonatal mouse brain in vivo.

Materials and Methods

Animals

Wild-type C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in the laboratory. Animals were euthanized and brain tissue harvested at different postnatal days for gene expression analyses. For gene expression analyses and ChIP assays neonatal mice [postnatal day (P) 4] were given ip injections of saline or 3.5,3’-triiodothyronine (T₃, sodium salt; 25 µg/kg body weight; Sigma, St. Louis MO), euthanized 4 h later, and blood plasma and brain tissue harvested for analysis. All procedures were conducted in accordance with guidelines of The University of Michigan Committee on the Care and Use of Animals.

RNA isolation and gene expression analysis by quantitative, real-time RT-PCR

We extracted RNA from microdissected mouse brain sections that contained either the hippocampus or cerebellum or from N2a[TRβ1] cells using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. The RNA was treated with deoxyribonuclease I and ribonuclease inhibitor before cDNA synthesis with Superscript II (Invitrogen) using random hexamers (Applied Biosystems, Foster City, CA). TaqMan assays (Applied Biosystems) were used to quantify transcripts for Klf9 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For Klf9 we designed a custom TaqMan assay to span the exon/exon boundary (Klf9 has two exons and a single intron; supplemental Table 1, published as supplemental data) encompassing 10 kb upstream of the transcription start site; see Table 1) and pGL3-Klf9[DR-4T₃RE] (corresponding to the DR-4 T₃RE located at −3.8 kb upstream of the transcription start site; see supplemental Table 2). The pκDR-4Luc plasmid contains the rat growth hormone T₃RE and was a gift of Ronald Koenig (University of Michigan, Ann Arbor, MI).

EMSA

We conducted EMSA as described by Hoopfer et al. (12) with recombinant TRα, TRβ, and retinoid X receptor (RXR)-α produced in vitro using the TnT SP6 quick-coupled translation system (Promega) following the manufacturer’s instructions. The ability of the wild-type or mutant mKlf9[DR-4 T₃RE] to displace TRβ-RXR binding to the [³²P]mKlf9[DR-4 T₃RE] probe was tested by competitive EMSA.

Cell culture and transfection assay

We used the mouse neuroblastoma cell line Neuro-2a that was engineered to express TRβ1 [N2a (TRβ1); 17] to investigate gene expression and TR association and chromatin modifications at putative T₃REs in the mouse genome. This cell line was previously shown to up-regulate Klf9 mRNA after T₃ treatment (13). Cells were plated at a density of 0.5 × 10⁵ cells/well in six-well plates for gene expression analyses, 1 × 10⁵ cells/well in 24-well plates for transfection assays, or 2.5 × 10⁴ in 100-mm tissue culture dishes for ChIP assays and cultured overnight before transfections or hormone treatments. Cells were cultured at 37 °C in an atmosphere of 5% CO₂–95% air in 50:50 DMEM-Hams F12 containing 500 µg/ml hygromycin B, penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and 10% thyroid hormone-stripped (25) fetal bovine serum (Invitrogen). For gene expression analyses in N2a[TRβ1] cells, the medium was switched to serum-free DMEM-F12 and cells were treated with T₃ for 6 h before harvest and RNA isolation.

For luciferase reporter assay experiments, we transfected cells with enhancer-reporter plasmids (200 ng DNA per well) using the FuGene 6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer’s instructions. All cells were cotransfected with the pRenilla-luciferase plasmid (10 ng/well; Promega) for normalization of cell transfection by dual-reporter luciferase assay following the manufacturer’s instructions (Promega). Immediately before transfection the cells were washed twice with serum-free DMEM-F12. After 1 h the transfection medium was replaced with growth medium and the cells were incubated overnight. Cells were then treated with or without T₃ for different times before harvest and analysis of luciferase activity. Luciferase activity was quantified (measured as relative light units) using a luminometer (Femtomater FB 12; Zylux Corp., Maryville, TN). Each transfection experiment was conducted four times with three to six wells per treatment.

ChIP assay

We conducted ChIP assays on chromatin extracted from tissue culture cells and mouse brain following methods described previously with slight modifications (26, 27). We used the ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) following the manufacturer’s instructions. To cross-link nuclear proteins, cells or brain lysates were treated with the homobifunctional cross-linking agent dithiobis(succinimidyl propionate) (DSP; Pierce Chemical Co., Rockford, IL) (28). Cells in 100-mm dishes were washed with Dulbecco’s PBS (DPBS; pH 7.4); In-vitrogen), 10 ml of DPBS were added, and a 25-mlm solution of DSP.
TABLE 1. Comparison of several DR-4 T3REs with the predicted mouse, human, and rat KLF9 DR-4 T3REs

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Species/gene</th>
<th>Genomic position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGTCanntaAGGTCA</td>
<td>Optimal DR-4 T3RE</td>
<td>2584 to 2599</td>
</tr>
<tr>
<td>AGGTGaaatAGGTCA</td>
<td>mKLF9 DR-4 T3RE</td>
<td>−3830 to −3804&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGATTCgttcAGGTCA</td>
<td>hKLF9 DR-4 T3RE</td>
<td>−2891 to −2875&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGTTGgctcAGGTCA</td>
<td>rKLF9 DR-4 T3RE</td>
<td>−3837 to −3819&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGACCttgaaAGGTCA</td>
<td>mIntronic IP-T3RE</td>
<td>+5159 to +5175&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGGTCanntaAGGTCA</td>
<td>Optimal DR-4 T3RE</td>
<td>2584 to 2599</td>
</tr>
<tr>
<td>AGTTCatctAGGACA</td>
<td>xKLF9 DR-4 T3RE</td>
<td>−6500&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGGCCAtctcAGGACA</td>
<td>hr DR-4 T3RE</td>
<td>−2599 to −2584&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGGCCTctctAGGTCA</td>
<td>rPCP-2 DR-4 T3RE A1</td>
<td>−295 to −268&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGGGCGtcctAGGGAA</td>
<td>rPCP-2 DR-4 T3RE A2</td>
<td>+207 to +227&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAATTaaagAGGTAA</td>
<td>hRRC3/neurogranin DR-4 T3RE</td>
<td>+3000 (first intron)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The <sup>a</sup> number of base positions upstream (−) or downstream (+) of the transcription start sites.

<sup>b</sup> This study. The rat and human T3REs are located in genomic regions homologous to the region of the mouse T3RE described in this study.

<sup>c</sup> (18).

<sup>d</sup> This DR-4 T3RE was reported by Thompson and Bottcher (37) to be at about −9kb in the rat hr gene. Engelhard and Christiano (39) later localized this sequence to −2599 to −2584 upstream of the mRNA cap site. This putative DR-4 T3RE is 100% conserved with human (39) and mouse (located −1921 to −1906; this study). The hr DR-4 T3RE region analyzed in our study is that reported by Engelhard and Christiano (39), who demonstrated its functionality using transfection assays.

<sup>e</sup> (40).

<sup>f</sup> (41).

Dissolved in dimethyl sulfoxide was added dropwise to a final concentration of 0.2 mM. The cells were incubated for 15 min at room temperature, the medium removed, and the cells washed with DPBS before proceeding to formaldehyde cross-linking. For the mouse brain, 0.3–0.5 mg of tissue was homogenized using a Dounce homogenizer in 0.75 ml nucleic extraction buffer [0.5% Triton X-100, 10 mM Tris-HCl, (pH 7.5), 3 mM CaCl<sub>2</sub>, 0.25 mM sucrose, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail; Sigma-Aldrich]. The stock DSP solution was added dropwise to a final concentration of 0.2 mM, and the samples were incubated at room temperature with rotation for 20 min. The samples were centrifuged at 2000 × g at 4°C for 2 min and the pellets washed with DPBS and resuspended in 1 ml nucleic extraction buffer. Twenty-five microliters of 37% formaldehyde were added to cross-link proteins to DNA.

The chromatin extraction procedure and DNA shearing by sonication were optimized for N2a[TR] extraction buffer. Twenty-five microliters of 37% formaldehyde were added dropwise to a final concentration of 0.2 mM. The cells were incubated for 15 min at room temperature, the medium removed, and the cells washed with DPBS before proceeding to formaldehyde cross-linking. For the mouse brain, 0.3–0.5 mg of tissue was homogenized using a Dounce homogenizer in 0.75 ml nucleic extraction buffer [0.5% Triton X-100, 10 mM Tris-HCl, (pH 7.5), 3 mM CaCl<sub>2</sub>, 0.25 mM sucrose, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail; Sigma-Aldrich]. The stock DSP solution was added dropwise to a final concentration of 0.2 mM, and the samples were incubated at room temperature with rotation for 20 min. The samples were centrifuged at 2000 × g at 4°C for 2 min and the pellets washed with DPBS and resuspended in 1 ml nucleic extraction buffer. Twenty-five microliters of 37% formaldehyde were added to cross-link proteins to DNA.

For ChIP assay we used a rabbit polyclonal antiserum raised against the full-length Xenopus laevis TRβ (PB antiserum provided by Yun-Bo Shi; National Institute of Child Health and Development, National Institutes of Health, Bethesda, MD). This antiserum has been used extensively for ChIP assay on frog tissues [e.g. Refs. 26, 29, 30], and the frog and mouse TR proteins share greater than 90% sequence identity. This antiserum does not distinguish TRα from TRβ. For acetylated histones 3 (acH3) and 4 (acH4), we used polyclonal antisera against Upstate Biotechnology; α-acH3, catalog no. 06-599; α-acH4 cat. no. 06-598; Upstate Biotechnology). To determine nonspecific binding (NSB), we either used straight normal rabbit serum (NRS; Sigma-Aldrich; for experiments with the PB antiserum) or we purified the IgG from NRS using a protein A column following the manufacturer’s instructions (Pierce; for acetylated histones). The degree of enrichment in the ChIP assay for anti-TR relative to the NRS varied, depending on the treatment (≥ T<sub>1</sub>) and the genomic region analyzed and ranged from about 1.5- to 7-fold. For anti-acH3 or anti-acH4, the degree of enrichment ranged from about 30- to greater than 350-fold. Negative controls for the ChIP assays included the omission of primary antibody (which uniformly produced no signal; data not shown), replacement of the primary antibody with NRS (straight or purified IgG), and the analysis of regions outside of the predicted T3RE regions (the proximal Klf9 promoter, the medial intron at 11.5 kb downstream of the transcription start site). ChIP samples were analyzed by real-time quantitative PCR using TaqMan assays (supplemental Table 2). Standard curves were constructed using a pool of input samples, and each ChIP sample was normalized to its respective input.

Data analysis and statistics

Data were analyzed by one-way ANOVA or t test using the SYSTAT computer program (version 10; SPSS Inc., Chicago, IL). Data were log<sub>10</sub> transformed if the variance was found to be heterogeneous by Bartlett’s test. Percentages are shown in the figures; percentages were arcsine transformed before statistical analysis. P < 0.05 was accepted as statistically significant. Gene expression data are reported as the mean ± SEM.

Results

Klf9 mRNA exhibits a postnatal rise in expression in mouse brain and is induced by thyroid hormone

Quantitative real-time RT-PCR analysis showed that Klf9 mRNA in the mouse hippocampal region and cerebellum began to rise between postnatal d 4 and 7 and reached a peak at d 14 that was maintained through d 30 [Fig. 1A; hippocampus: F(4, 19) = 10.447, P < 0.0001; cerebellum: F(4, 19) = 17.286, P < 0.0001; one way ANOVA]. A similar pattern of gene expression was observed with hsv mRNA [Fig. 1B; hippocampus: F(4, 17) = 7.923, P = 0.002; cerebellum: F(4, 19) = 32.805, P < 0.0001; cerebellum
potential inverted palindrome T3RE with half-sites spaced by 97 and 85%, respectively, with the mouse gene. We located a minimal promoters (500 bp) of the rat and human sequence identity, respectively, with the mouse gene. The proximal promoters (200 bp segment encompassing the putative T3RE) were also present in the rat and human Klf9 genes. For comparison, the entire introns of the rat and human Klf9 genes share 84 and 59% sequence identity, respectively, with the mouse gene.

EMSA showed that TR-RXR heterodimers bound to the mouse Klf9 T3RE (Fig. 2, B and C). The binding of both TR-α-RXRs and TRβ-RXRs heterodimers was reversible and of high affinity; TR monomers or homodimers did not bind. Binding of the protein complexes to the X. laevis TRβ T3RE (xtT3RE) is shown for comparison to the left on the gel in Fig. 2B. Competitive binding assays with the native mouse Klf9 T3RE showed a DNA binding affinity in the low nanomolar range (~5 nM), whereas a mutated mKlf9 T3RE (see supplemental Table 2) did not displace the radiolabeled probe (Fig. 2C). TR-RXR heterodimers also bound to the upstream rat and human DR-4 T3REs and the mouse intronic IP-4 T3RE (Fig. 2D).

The Klf9 DR-4 T3RE supports T3-dependent transactivation

We tested for functionality of the mouse Klf9 DR-4 T3RE by transfection assay using putative T3RE sequences subcloned into a minimal promoter reporter plasmid (pGL3promoter). The pGL3mKlf9[DR-4T3RE] plasmid transfected into N2a[TRβ1] cells was activated by T3 in a time-dependent manner, with increased luciferase expression at 6 h and maximum expression at 24 h [4.6-fold; Fig. 3A; F(3, 21) = 58.575, P < 0.0001, ANOVA]. The ptkDR-4Luc plasmid included as a positive control was similarly activated by T3 [first significant elevation at 3 h, maximum activation at 24 h, 7.2-fold; F(3, 21) = 45.105, P < 0.0001]; luciferase activity in cells transfected with empty vector (pGL3promoter) was not altered by T3 treatment (data not shown).

In contrast to the native Klf9 DR-4T3RE, cells transfected with a plasmid containing the mutated mouse Klf9 DR-4T3RE (pGL3mKlf9[mtDR-4T3RE]) did not show activation of luciferase expression by T3 treatment (for 24 h; Fig. 3B; pGL3mKlf9[DR-4T3RE] 5.2-fold activation, P < 0.0001; t test; ptkDR-4Luc 8-fold activation, P < 0.0001). Cells transfected with the plasmid containing the mutant T3RE had a higher basal level of luciferase expression, which likely reflects the removal of the repressor activity of the unliganded TR (which represses basal activity of pGL3mKlf9[DR-4T3RE] in the absence of T3). The putative mouse intronic IP-4T3RE and rat DR-4T3RE were also activated by T3, although to a lesser extent than the mouse DR-4T3RE (~2-fold; pGL3mKlf9[intronicIP-4T3RE] control, 10.90 ± 0.54; T3, 25.73 ± 1.81, P < 0.001, t test; pGL3rKlf9[DR-4T3RE] control, 4.58 ± 0.67, T3, 8.45 ± 0.27, P < 0.0001); we did not test the putative human DR-4 T3RE in transfection assay.

TRs associate with the Klf9 DR-4 T3RE region in N2a[TRβ1] cells as analyzed by ChIP assay

Treatment of N2a[TRβ1] cells with T3 caused a dose-dependent increase in Klf9 and br mRNA levels (see supplemental
Thyroid hormone treatment of N2a[TRβ1] cells increases acetylation of histones 3 and 4 at the Klf9 DR-4 T3RE

Basal levels of acetylation of histones 3 and 4 were comparable across genomic regions analyzed (Fig. 4, B and C). Treatment of N2a[TRβ1] cells with T3 increased acetylated histone 3 \( [F(7, 46) = 23.078, P < 0.0001; \text{ANOVA}] \) and histone 4 \( [F(7, 46) = 30.166, P < 0.0001] \) ChIP signals at several Klf9 genomic regions with the largest increase at the DR-4 T3RE (acH3, 3.3-fold; acH4, 5.9-fold). There was no significant change in either AcH3 or acH4 at the proximal promoter region. Similar increases were observed at the br DR-4 T3RE region (acH3, 2.7-fold; acH4, 3.3-fold; \( P < 0.0001 \) for both; \( t \) test).

TR associates with the Klf9 DR-4 T3RE in neonatal mouse brain in vivo

We observed a significant TR ChIP signal when compared with NRS controls at the Klf9 DR-4 T3RE in both hippocampus (NRS = 0.07%, TR = 0.17%, \( P = 0.008; \) \( t \) test) and cerebellum (\( P = 0.01; \) Fig. 5A) of neonatal mice. No significant TR ChIP signal was observed at the proximal promoter, proximal intron, or medial intron (only cerebellum analyzed). By contrast, we did not detect TR ChIP signal at the br DR-4 T3RE (data not shown).

We found significant acH4 ChIP signal compared with NRS controls at each region of the mouse Klf9 gene except at the medial intron in hippocampus and cerebellum (\( P < 0.05; \) \( t \) tests; Fig. 5, B and C; only acH4 was analyzed due to limitation in the amount of available chromatin). There were significant differences in normalized acH4 ChIP signal across the Klf9 gene in both hippocampus \([F(3, 15) = 4.361, P = 0.027]\) and cerebellum \([F(3, 15) = 4.167, P = 0.031]\) with the highest signal at the Klf9 DR-4 T3RE. A similar high level of acH4 signal was observed at the br DR-4 T3RE in both brain regions (acH4 \textit{vs.} NRS: hippocampus, \( P = 0.01; \) cerebellum, \( P = 0.029; \) \( t \) test).
Kruppel-like factor 9 promotes differentiation of mammalian and amphibian neuronal cells, mediating T₃ actions on neurite extension and branching (13–15). Disruption of the Klf9 gene in mouse led to behavioral abnormalities characteristic of defects in the hippocampus, cerebellum, and amygdala and reduced dendritic arborization of cerebellar Purkinje cells (16). The Klf9 gene was isolated as a direct T₃ response gene in the frog (11, 31), and expressed as a percentage of the input minus the NSB. Statistical analyses were conducted on arcsine transformed data, and letters indicate significant differences among means (P < 0.05; Fisher’s least squares difference test; n = 6/treatment). *, Statistically significant differences (P < 0.05; Student’s unpaired t test; n = 6/treatment).

The rapid kinetics of Klf9 up-regulation by T₃ in N2a[TRB1] cells, the fact that T₃ induction of Klf9 mRNA is resistant to protein synthesis inhibition, and the fact that nuclear run-on assay showed that T₃ initiates Klf9 transcription (13) suggested that mouse Klf9 is a direct T₃ response gene. Furlow and Kanamori (18) provided evidence for a DR-4 T3RE located about 6.5 kb upstream of the transcription start site of the frog Klf9 gene. We therefore searched for T3REs in the mouse Klf9 gene to explain its regulation by T3. TRs bind to DNA at the consensus hexamer sequence (G/A)GGT(C/G)A, referred to as a direct repeat (DR-4) (24), and the preferred configuration is a heterodimer with RXR (24), spaced by four to six nucleotides or palindromes (Pal) (reviewed in Ref. 24).

FIG. 3. The mouse Klf9 DR-4 T₃RE confers thyroid hormone responsiveness to a minimal, heterologous promoter. A, Treatment with T₃ (30 nM) increases luciferase activity in cells pGL3mKlf9[DR-4T₃RE] in a time-dependent manner. N2a[TRB1] cells were transiently transfected with pGL3mKlf9[DR-4T₃RE] or ptkDR-4Luc (rat GH T₃RE; positive control) and treated with T₃ for the times indicated before harvest and analysis of cell lysates by dual-luciferase assay. Letters indicate significant differences among means (P < 0.05; Fisher’s least squares difference test). B, Mutation of the mouse Klf9 DR-4T₃RE eliminates T₃-dependent transactivation in transfection assay. N2a[TRB1] cells were transiently transfected with pGL3mKlf9[DR-4T₃RE], pGL3mKlf9[mtDR-4T₃RE] (mutated T3RE) or ptkDR-4-Luc and treated with T₃ (30 nM) for 24 h. Luciferase activity in cells transfected with empty vector (pGL3promoter) was not altered by T₃ treatment (data not shown). *, Statistically significant differences (P < 0.05; Student’s unpaired t test).

FIG. 4. A, TR associates with the mouse Klf9 DR-4T₃RE and the hr DR-4T₃RE in N2a[TRB1] cells as analyzed by ChIP assay. N2a[TRB1] cells were treated with or without T₃ (30 nM) for 24 h before harvest for ChIP assay. ChIP samples were analyzed by real-time quantitative PCR using TaqMan assays that targeted the genomic regions indicated (see supplemental Table 2). The TR ChIP signals at the Klf9 DR-4T₃RE and the hr DR-4T₃RE were significantly increased by T₃ treatment. Treatment with thyroid hormone causes hyper-acH3 (B) and -acH4 (C) in N2a[TRB1] cells as analyzed by ChIP assay. NSB was assessed by ChIP using NRS (A) or IgG purified from NRS (B and C). Shown are the mean ChIP signals expressed as a percentage of the input minus the NSB. Statistical analyses were conducted on arcsine transformed data, and letters indicate significant differences among means (P < 0.05; Fisher’s least squares difference test; n = 6/treatment). *, Statistically significant differences (P < 0.05; Student’s unpaired t test; n = 6/treatment).
two sequences based in part on the strong conservation of the genomic regions in which they were found among mouse, rat, and human, one at −3.8 kb and one within the intron at about +5.2 kb. The −3.8 kb T3RE is of the type DR-4 with head-to-tail orientation of half-sites, whereas the intronic T3RE has the configuration of an inverted palindrome with half-sites spaced by four nucleotides (IP-4). Other putative T3REs found in the upstream region or intron by computer analysis were represented by single half-sites [T3REs of almost all positively regulated genes are comprised of two or more half-sites (24)], poor matches to known T3REs, or were not conserved across species and thus were not studied further. We cannot rule out the possibility that other functional T3REs not identified by this approach that may influence Klf9 expression are present within, near, or far upstream of the Klf9 locus, and further study is required to test this.

We found that both putative mouse Klf9 T3REs were bound by TR-RXR heterodimers in gel shift assays and that both supported T3-dependent transactivation in transfection assay. However, whereas TR was found to be associated with the −3.8 kb DR-4 T3RE in both N2a[TRβ1] cells and mouse brain in vivo by ChIP assay, we found no association of TR with the putative intronic IP-4 T3RE region, thus failing to support that this sequence is a functional T3RE. This finding illustrates that, although a putative T3RE may be bound by TR-RXR in vitro and may support T3-dependent transcription in transfection assay, analysis of TR association with the genomic region in vivo by ChIP assay is necessary to test whether the element is functional. Treatment of N2a[TRβ1] cells with T3 increased the ChIP signal at both the Klf9 and br upstream DR-4 T3RE regions but not at the proximal promoter, proximal intron, or medial intron of the Klf9 gene. The increased TR signal at the upstream Klf9 DR-4 T3RE (and the br DR-4T3RE) further supports that this region possesses a bona fide hormone response element. In the unliganded state, TRs are associated with chromatin and are not thought to be recruited to genomic sites on hormone binding as occurs for some other members of the steroid receptor superfamily (32). By contrast, we found evidence for recruitment of TRs to T3REs upon hormone binding. Because the expression of TRβ1 in N2a[TRβ1] cells is maintained at a constant level through stable transfection (17) and we previously showed that T3-dependent Klf9 expression is mediated by TRβ1 and not TRα1, it is unlikely that the increased TR ChIP signal was due to increased TR biosynthesis. Buchholz et al. (30) reported that TR association with the Xenopus TRβ and basic leucine zipper transcription factor (TH/bZip) promoters in vivo was increased by treatment of tadpoles with T3. This increase could have been due to T3-dependent recruitment of TRs to genomic sites or perhaps as a result of increased TR biosynthesis, which occurs in tadpoles [TRβ autinduction; (33)]. Our findings suggest that whereas TR associates with T3REs in the genome in the unliganded form, additional TR recruitment to T3REs may occur on ligand binding in vivo. An alternate explanation is that ligand binding to TR or histone modifications at the T3RE caused by T3-dependent recruitment of coregulators to this site may expose epitopes on TR, thus resulting in more efficient ChIP. Further study is required to distinguish these potential mechanisms.

![FIG. 5.](image)

A, TR associates with the mouse Klf9 DR-4 T3RE in neonatal mouse cerebellum as analyzed by ChIP assay. P4 mice were injected with T3 (25 μg/kg body weight) and brain tissue harvested 4 h later for chromatin extraction and ChIP assay (see Materials and Methods). acH4 is elevated at the Klf9 DR-4 T3RE and the br DR-4 T3RE in mouse hippocampus (B) and cerebellum (C) as analyzed by ChIP assay. ChIP samples were analyzed by real-time quantitative PCR using TaqMan assays that targeted the genomic regions indicated in the graph (and supplemental see Table 2). Shown in the graphs are the mean ChIP signals normalized to input for PB anti-TR serum (anti-TR) or NRS. However, statistics were conducted on the normalized TR or acH4 ChIP values, i.e. the mean ChIP signals expressed as a percentage of the input minus the NSB. NSB was assessed by ChIP using NRS (A) or IgG purified from NRS (B and C) for anti-TR, the ChIP signal was significantly greater than for NRS only at the DR-4 T3RE (\(P < 0.05\); Student’s unpaired t test; \(n = 4\)). For anti-acH4, the ChIP signal was significantly greater than for NRS IgG at all regions of the Klf9 gene except the medial intron; statistics shown in the graphs do not compare the mean anti-acH4 vs. NRS ChIP signals but instead the normalized anti-acH4 ChIP values among Klf9 gene regions (letters indicate significant differences among means; \(P < 0.05\); Fisher’s least squares difference test; \(n = 4\)). Anti-acH4, Purified IgG to acetylated histone 4.

as we also found in our gel shift assays using the mouse Klf9 DR-4T3RE (see Fig. 2). TR-RXR heterodimers exhibit a strong preference for DR-4, and DR-4T3REs are the most common and best-characterized response elements (24). Studies in the frog X. laevis using ChIP assay confirmed that TR-RXR is associated with DR-4 elements in the TRβ and basic leucine zipper transcription factor (TH/bZip) gene promoters in vivo during tadpole metamorphosis (26).

Using computer analysis, we identified putative T3REs upstream and within the mouse Klf9 gene. We chose for analysis
The TR-RXR heterocomplex recruits coregulator proteins that mediate the repressive or activational actions of the complex by recruiting histone modifying enzymes such as histone acetylases and histone deacetylases, among others (34, 35). We observed robust increases in the acetylation of histones 3 and 4 at the upstream Klf9 DR-4T(RE) caused by T3 treatment in N2a[TRβ1] cells and significantly greater acH4 at the DR-4T(RE) compared with other regions of the Klf9 gene in mouse brain in vivo. The level of histone acetylation was greatest at the upstream DR-4T(RE), but elevated histone acetylation caused by T3 treatment was observed at all regions of the Klf9 locus analyzed. The widespread histone acetylation could be due to spreading of histone modifications across the genetic locus originated from the site of TR binding. Interestingly, histone acetylation at the proximal Klf9 promoter was the least affected by T3.

Parker et al. (36) recently reported in Drosophila cells that, on activation of Wnt signaling, acetylation of histones 3 and 4 spread over relatively large genomic regions occupied by the Wnt target genes naked cuticle and Notum, with relatively small changes occurring at the promoter regions of these genes. By contrast, histone acetylation was restricted to the promoter regions of housekeeping genes that were not activated by Wnt. Whether such spread of histone acetylation, with the hormone response element acting as a nucleation site, is a characteristic of hormone-responsive genes requires further study.

The hr gene encodes a zinc-finger domain transcription factor that interacts with TRs and function as a corepressor (37, 38). The hr mRNA exhibited developmental stage and T3-dependent regulation in mouse brain similar to that of Klf9. Also, we found TR association and T3-dependent histone acetylation with the predicted hr DR-4T(RE) located at about 2 kb. Although we found TR association with the hr DR-4T(RE) in N2a[TRβ1] cells, we were unable to confirm this in mouse brain in vivo. Thus, our evidence supports that the mouse hr gene has a functional T3(RE) at about 2 kb upstream of the transcription start site, but further study is required to elucidate its role in mouse brain in vivo.

Taken together, our findings support the presence of a functional DR-4 T(RE) at 3.8 kb upstream of the transcription start site of the mouse Klf9 gene to explain its regulation by T3 during early postnatal life. We also provide evidence that this T3(RE) may be evolutionarily conserved among mammals. We found that TRs may be recruited to chromatin on ligand binding, and future studies should analyze dynamic changes in TR recruitment, corepressor/coactivator exchange, and histone modifications at the mouse Klf9 gene and other T3-responsive genes in vivo.

Acknowledgments

We are grateful to Yun-Bo Shi for providing the PB antiserum and Anna Ray and Jiefei Geng for technical assistance. We are also grateful to Pia Bagamasbad for conducting the quantitative RT-PCR analyses for hr mRNA. Ron Koenig kindly provided the ptkDR-4Luc and the TR and RXR expression plasmids. Jack Puymirat provided the N2a[TRβ1] cells.

Address all correspondence and requests for reprints to: Dr. Robert J. Denver, Department of Molecular, Cellular, and Developmental Biology, The University of Michigan, Ann Arbor, Michigan 48109-1048. E-mail: rdenver@umich.edu.

This work was supported by National Institutes of Health Grant 1R01NS046690-01 (to R.J.D.).

Disclosure Summary: The authors have nothing to disclose.

References

5. Gould E, Allan MD, McEven BS 1990 Dendritic spine density of adult hippocampal pyramidal cells is sensitive to thyroid hormone. Brain Res 527:327–329
18. Furlow JD, Kanamori A 2002 The transcription factor basic transcription element-binding protein 1 is a direct thyroid hormone response gene in the frog Xenopus laevis. Endocrinology 143:3295–3305

3942

Endocrinology, August 2009, 150(8):3935–3943

Denver and Williamson A Functional DR-4 T3RE in the Mouse Klf9 Gene

Downloaded from endo.endojournals.org at Univ Of Mich Library on July 22, 2009
24. Yen PM 2001 Physiological and molecular basis of thyroid hormone action. Physiol Rev 81:1097–1142
34. Wu Y, Koenig RJ 2000 Gene regulation by thyroid hormone. Trends Endocrin Metab 11:207–211
37. Thompson CC, Bottcher MC 1997 The product of a thyroid hormone-responsive gene interacts with thyroid hormone receptors. Proc Natl Acad Sci USA 94:8527–8532