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Characterization of the 5′-Flanking and 5′-Untranslated Regions of the Cyclic Adenosine 3′,5′-Monophosphate-Responsive Human Type 2 Iodothyronine Deiodinase Gene*

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ABSTRACT

The type 2 iodothyronine deiodinase (D2) catalyzes T_4 activation. In humans, unlike rodents, it is widely expressed, and its action probably contributes to both intracellular and plasma T_3 pools. We have isolated the 6.5-kb 5′-flanking region (FR) and the previously uncloned 553 nucleotides (nt) of the 5′-untranslated region (UTR) of h*dio*2. The 5′-UTR is complex, with three transcription start sites (TSS) (708, 31, and ~24 nt 5′ to the ATG), an alternatively spliced approximately 300-nt intron in the 5′-UTR, and three short open reading frames 5′ to the initiator ATG. The previously reported approximately 7.5-kb D2 messenger RNA (mRNA) is actually an approximately 7-kb doublet that is present in thyroid, pituitary, cardiac and skeletal muscle, and possibly brain, but with only the longer transcript in placenta. A canonical cAMP response element-binding protein-binding site is present at about 90 bp 5′ to the most 5′-TSS. It accounts for the robust response of the 6.8-kb h*dio*2 5′-FR to protein kinase A. Forskolin increases D2 mRNA in human thyroid cells, which may explain the high D2 mRNA in Graves' thyroid and thyroid adenomas. The h*dio*2 gene structure and Northern blot results suggest that D2 expression is tightly controlled and tissue specific. (*Endocrinology* **140:** 229–237, 2000)

A PARTIAL complementary DNA (cDNA) containing the complete coding sequence of the human type 2 iodothyronine deiodinase (D2) gene (h*dio*2) has recently been identified (1, 2).[1] More recently, using homology searches of the GenBank EST database, we have identified two additional overlapping fragments that comprise the 3′-untranslated region (3′-UTR) of the hD2 cDNA, including the SECIS element (3). The remaining 5′-UTR has not been cloned. A surprising aspect of the early studies of this cDNA was that human D2, unlike that of the rat, is highly expressed in thyroid tissue and in skeletal and cardiac muscle (1, 2, 4). The expression of D2 activity in the large mass of skeletal muscle suggests the possibility that in humans, the T_3 derived from the 5′-deiodination of T_4 catalyzed by this deiodinase might contribute significantly to the plasma T_3 pool as well as provide intracellular T_3 (1, 2). The expression of D2 in human thyroid was also unexpected, in that this enzyme, unlike type 1 iodothyronine deiodinase (D1), is not present in rat-derived FRTL5 cells (4, 5). Furthermore, D2 messenger RNA (mRNA) or its activity was significantly elevated in thyroid tissue from patients with Graves' disease, TSH-induced hyperthyroidism, or thyroid adenomas (5). This suggested that the human *dio*2 gene responded to thyroid stimulation, possibly through cAMP.

In rats, activation of the sympathetic nervous system by cold exposure increases D2 mRNA and activity in brown adipose tissue (BAT), an effect that can be blocked by α_1- or β-adrenergic blocking agents (1, 6). These agents also increase D2 activity in isolated brown adipocytes by an actinomycin D-sensitive mechanism (7). D2 mRNA is also increased by forskolin or 8-bromo-cAMP in rat astroglial cells (8), explaining the rapid cAMP stimulation of D2 activity in these cells (9). Taken together, these results suggest the presence of a cAMP response element (CRE) in the rat as well as in the human *dio*2 gene.

The goal of the present studies was to analyze the promoter, 5′-flanking and 5′-UTR of h*dio*2. We found that two major transcripts (~6.8 and 7.5 kb) are expressed in thyroid and muscle and probably brain, with only a single 7.5-kb transcript present in placenta. We identified three transcriptional start sites (TSS), 708, 32, and 25 nucleotides (nt) 5′ to the initiator methionine, and an alternatively spliced intron in the 5′-UTR. A single functional CRE is present in the 6.5-kb 5′-flanking region about 70 bp 5′ to the upstream TATA sequence, accounting for the increased D2 mRNA expression

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[1] The promoter and 5′-flanking sequences have been submitted to GenBank (AF188709).

in Graves' or TSH- or forskolin-stimulated human thyroid cells.

Materials and Methods

Isolation and characterization of a human dio2 promoter region gene

A human genomic library (λ-Fix II vector, Stratagene, La Jolla, CA) was screened by filter hybridization with a probe from the 5′-portion of the human cDNA Z44085 (nt −40 to +302) (2) using standard techniques as previously described (10). This clone was provided by D. St. Germain and V. Galton (1). Two positive clones were mapped by Southern hybridization after digestion with various restriction enzymes. One of these, clone 2, was plaque purified, and the *Not*I fragment was subcloned into a pBluescript plasmid (pBS, Stratagene) using standard techniques (10). It was partially sequenced and extends approximately 7 kb 5′ to the initiator ATG codon.

RNA isolation and Northern blotting

One to 2 g of several human thyroid glands obtained at surgery were homogenized in liquid N_2 in a mortar. The homogenized tissues were transferred to a 15-ml tube and lysed in Trizol (Life Technologies, Inc., Grand Island, NY). Total RNA was isolated according to the manufacturer's protocol. Twenty micrograms of total RNA were denatured and electrophoresed on a 2.2 M formaldehyde-1% agarose gel in 1 × MOPS buffer and transferred to nylon membranes (GeneScreen, DuPont, Wilmington, DE) as previously described (4). We also purchased a multiblot from CLONTECH Laboratories, Inc. (Palo Alto, CA), containing 2 μg polyadenylated [poly(A)$^+$] mRNA from various human tissues.

Membranes were prehybridized in 5 × SSPE, 50% deionized formamide, 5 × Denhardt's solution, 1% SDS, 10% dextran sulfate (mol wt, 500,000), and 100 μg/ml denatured sheared calf thymus DNA at 42 C for 3 h. cDNA probes were labeled with [α-^{32}P]deoxy (d)-CTP and random primers using Prime-It II kit (Stratagene). For detection of the coding region, an hD2 coding region fragment (708–1531 nt 3′ to the TSS) was used. For hybridizations to identify the 5′-portion of the 5′-UTR, a PCR fragment containing nt 18–321 was used, and to identify a putative 5′-intron, a PCR-generated fragment containing nt 352–645 was used. Hybridizations were performed with 1×10^6 cpm/ml hybridization mix of denatured labeled cDNA probe at 42 C for 16–24 h in a rotating bottle of the hybridization oven. Membranes were washed with 2 × SSC (standard saline citrate)-0.1% SDS at room temperature, 1 × SSC-0.1% SDS at 42 C and finally with 0.25 × SSC-0.1% SDS at 42 C. The membrane was exposed to x-ray film for the indicated periods.

Primer extension

The 5′-end of the h*dio*2 gene transcript was determined using a primer extension system according to the manufacturer's protocol (Promega Corp., Madison, WI). To determine the 5′-end of the larger transcript, a 29-mer primer (16R) complementary to nt 225–253 (see Fig. 2 for primer locations) was 5′-end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA). Total RNA from human Graves' thyroid tissue was prepared using Trizol (Life Technologies, Inc.) and subsequently treated with ribonuclease-free deoxyribonuclease to remove residual genomic DNA. One hundred femtomoles of ^{32}P-labeled primer and 20 μg RNA were annealed in 1 × primer extension buffer [50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$,10 mM dithiothreitol (DTT), 1 mM dNTPs, and 0.5 mM spermidine] at 58 C for 20 min and cooled at room temperature for 10 min. Primer extension was performed by adding 2.8 mM sodium pyrophosphate and 0.4 U/μl AMV RT in 1 × primer extension buffer at 42 C for 30 min. The resulting product was analyzed on a 5% polyacrylamide-8.3 M urea gel in parallel with a sequencing reaction of hD2 genomic DNA.

Rapid amplification of 5′-cDNA ends (5′-RACE) reactions

An additional approach, 5′-RACE, was performed to define the more 3′-TSS using the manufacturer's protocol (Life Technologies, Inc.). Briefly, total RNA was isolated from thyroid tissue of a patient with hyperthyroidism secondary to a TSH-producing tumor (4). First strand synthesis and its poly(C)-tailing were followed the manufacturer's protocol from Life Technologies, Inc. Briefly, first strand cDNA was synthesized from 800 ng total RNA using SuperScript TM II reverse transcriptase (Life Technologies, Inc.) and a coding region-specific primer (9R) complementary to nt 1256–1288. The single strand products were poly(C)-tailed by terminal deoxynucleotide transferase and dCTP. Poly(C)-tailed cDNA was amplified by the Abridged Anchor Primer (AAP, 5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′) and an internal primer 4R (5′-AGC TAT CTT CTC CTG GGT ACC ATT GCC ACT GTTG-3′) complementary to nt 995-1028 (Fig. 2). PCR was performed with two different annealing temperature protocols. First, we denatured at 94 C for 2 min and then proceeded with two cycles consisting of denaturation at 94 C for 1 min, annealing at 55 C for 1.5 min, with amplification at 72 C for 2 min. Subsequently, 35 cycles of denaturation at 94 C for 1 min, annealing at 63 C for 50 sec, and amplification at 72 C for 1.5 min were performed. Lastly, extension was carried out at 72 C for 10 min. The PCR products were subcloned into pGem T-vector and sequenced using the Fidelity DNA sequencing system (Oncor, Inc., France).

To explore the possibility of an alternatively spliced intron in the 5′-UTR, two RT reactions were performed using two different protocols. In the first, 1 μg total RNA was mixed with 0.4 μg random hexamers, 0.5 mM dNTPs, 40 U RNasin, 5 mM DTT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 20-μl reaction volume. This was incubated at 37 C for 1.5 h. A second RT reaction was performed with Superscript RNase H$^-$-reverse transcriptase (Life Technologies, Inc.) at 50 C. Ten micrograms of total RNA were mixed with 10 pmol specific primer of 4R and 9R, 62.5 mM Tris-HCl (pH 8.3), 93.75 mM KCl, and 3.75 mM $MgCl_2$ in a 16-μl volume, which was preincubated at 65 C for 10 min and 62 C for 20 min, then cooled to room temperature for 10 min. Subsequently, 0.5 mM dNTPs, 40 U RNasin, 10 mM DTT, and 200 U Superscript RNase H$^-$-reverse transcriptase (Life Technologies, Inc.) were added, the final volume was made to 20 μl, and this was incubated at 50 C for 1.5 h. Subsequent PCR of both RT products was performed with 2 μl RT product using Vent polymerase according to the manufacturer's protocol (New England Biolabs, Inc.). PCR mixtures were denatured at 94 C for 4 min and then subjected to 30–33 cycles, consisting of denaturation at 94 C for 1 min, annealing at 58 C for 1 min with amplification at 76 C for 1.5 min, and extension at 76 C for 10 min.

S1 analysis of hD2 mRNA

S1 analysis was performed using synthesized, single stranded DNA probes as described previously (10). For determination of the second and third transcription start sites, a cDNA probe complementary to the RNA-coding strand, 716 5′-GGA TGC CCA TCT TCT CTG CCT CCT GAG TCA GTT CCC TTG TGC GCT CTG GTT CCC TAG TG-3′ 661 (TSS-2) was used. The 3′ four nucleotides were not complementary to the mRNA to permit S1 attack. For the alternatively spliced intron in the 5′-UTR, a probe was designed to detect the predicted 3′-intron boundary based on the RT-PCR results. This primer was complementary to nt 695 5′-CCT GAG TCA GTT CCC TTG TGC GCT CTG GTT CCC CTT CAC CCT CTT ATT TAA AAG GGC CCA-3′ 640 and again included four noncomplementary nucleotides (3′INT). Primers were 5′-end labeled using T_4 polynucleotide kinase. A mixture of 2 pmol of each primer, 150 μCi [α-^{32}P]ATP, 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, and 10 U T_4 polynucleotide kinase in 25 μl was incubated at 37 C for 1 h. The reactions were stopped by heating at 75 C for 10 min and purified through a Sephadex G-25 column.

For the hybridization, 5×10^4 cpm probe were mixed with 50 μg total RNA, which was then precipitated using ethanol. After washing the pellet with 70% ethanol, the pellet was dried inverted in air for 30 min. Then, the pellet was dissolved in 20 μl S1 hybridization solution [80% deionized formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA (pH 8.0)], denatured at 65 C for 10 min, and subsequently hybridized at 30 C overnight. For the S1 reaction, the hybridized RNA was incubated in 300 μl 280 mM NaCl, 50 mM sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$, 20 μg/ml single stranded calf thymus DNA, and 300 U S1 nuclease at 30 C for 1 h. The reaction product was precipitated by adding 80 μl S1 stop buffer [4 M ammonium acetate, 20 mM EDTA (pH 8.0), and 40 μg/ml transfer RNA], and 1 ml ethanol. After washing and drying the pellet, it was dissolved in 3 μl TE buffer and mixed with 17 μl

formamide loading dye. Ten microliters were loaded on a 6% polyacrylamide-urea gel to resolve the hybridized bands. This was subsequently exposed to BioMax x-ray film.

hD2 promoter-chloramphenicol acetyltransferase (CAT) constructs

Genomic fragments were cloned into polylinker sites of the pOCAT2 plasmid, a promoter insertion-CAT expression vector (11). Two *Pst*I sites were identified, 0.7 and 2.8 kb 5′ to the initiator ATG. The 3′-*Pst* site was at position +7 relative to the 5′-TSS (see *Results*). *Bam*HI sites were identified about 0.4 and 4.3 kb 5′ to the 5′-TSS (see Fig. 1). A 2.1-kb *Pst*I fragment was blunt ended and subcloned into the *Hin*cII site to make h*dio*2CAT3. A partial *Hin*dIII (3′) and *Sac*I (5′) fragment was then inserted into the *Sac*I/*Hin*dIII sites of h*dio*2CAT3. Restriction mapping showed that this construct was missing the two 3′-*Hin*dIII fragments (Figs. 1 and 2). To produce an intact 5′-flanking region construct, the internal (incomplete) 3.3-kb *Bam*HI fragment was deleted and replaced by the intact 3.9-kb *Bam*HI fragment cut from the genomic clone to form 6.5 h*dio*2CAT1. Several derivative constructs were prepared from hD2CAT1, including an internal *Bgl*II deletion (6.5 hdio2 (/δ*Bgl*II CAT); see Fig. 2), which removes nt −491 to −84.

Mutation of the CRE

Sequencing of the 5′-flanking region (5′-FR) identified a CRE sequence (TGACGTCA) located 94 bp 5′ to the major TSS. This site was modified by PCR mutagenesis to inactivate the CRE by changing the sequence to AGTCGACA. A mutated inner sense (CTCTTTCT-CAA[AGTCGACA]AGATCTTTAC) primer and an outer antisense primer (AGTTCTACTTTCATTTCCAAGCACCTATG) complementary to positions 293–321 of the 5′-UTR were amplified using the genomic clone 2 as a template in one reaction and an outer sense primer from position −633 to −612 (GAGCT CCTGT ATTCA AGTTT CTGCA AG) containing a 5′-*Sac*I restriction site together with the reverse complement of the mutant CRE oligonucleotide in the second. Vent polymerase (New England Biolabs, Inc.), which does not A-tail the products, was employed for these reactions to avoid unintended mutations. The mutant promoter/5′-FR was then reconstituted with *Taq* polymerase (Takara Biomedicals, Otsu, Japan) using the outer primers. A wild-type control was prepared using the same outer primers. The wild-type and mutant (mCRE) *Sac*I/blunt-ended *Pst*I fragments were then inserted into the *Sac*I/*Hin*cII-cut pOCAT2 vector. Both constructs were completely sequenced. The control and mutated constructs are designated h*dio*2wild-type (633)CAT and h*dio*2mutCRE (633)CAT, respectively.

Transient expression assays

Transfections of HEK-293 cells were performed with CaP-DNA precipitates using an internal TK human (h) GH control for transfection efficiency as previously described (12). CAT assays were performed by the method of Seed and Sheen (13), and results are expressed as the ratio of cellular CAT to medium hGH (14). cAMP responsiveness was tested by cotransfecting a plasmid that transiently expresses the a catalytic subunit of protein kinase A (PKA) under control of the Rous sarcoma virus promoter (provided by Dr. Richard Maurer) (15).

Human thyroid cell cultures

Cell cultures were prepared as previously described (16). Briefly, tissue from a thyroid nodule of a patient undergoing thyroidectomy for a toxic adenoma was cut into small pieces and digested by type IV collagenase (Sigma, St. Louis, MO; 125 mg/ml) in Ham's F-12 medium and 0.5% BSA overnight at 4 C in a rotating shaker. Cells were pelleted by centrifugation at 150 × *g* for 5 min, washed twice in BSA-Ham's F-12 medium, seeded in petri dishes, and cultured in 5% CO_2 atmosphere at 37 C in Ham's F-12 medium supplemented with 10% FCS. Medium was changed every 3 days, and subconfluent plates were used for treatment with forskolin (10 μM) (Sigma).

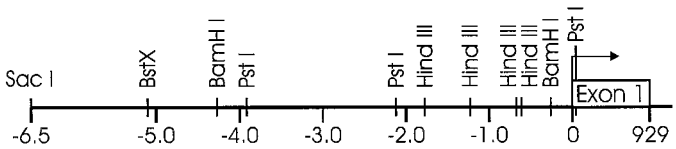

FIG. 1. Restriction map of the human *dio*2 gene promoter and 5′-flanking region.

Miscellaneous

All chemicals were of reagent grade and were obtained from commercial sources. All molecular biological manipulations were performed using standard techniques (10). Human tissues were obtained under an institutional review board-approved protocol.

Results

Defining the transcription start sites and structure of the hdio2 mRNAs

Clone 2 contained a genomic fragment of h*dio*2 that extended from a position approximately 6.5 kb 5′ to the TSS (see below) to an exon that included codons 1–74 and a fragment of the subsequent intron (Figs. 1 and 2). The sequence of an exon/intron junction at that position was 5′-349 GAG/GT (Fig. 2B). The sequence of the h*dio*2 gene from nt −650 to +1050 is shown in Fig. 2, together with the locations of introns, relevant restriction sites, and oligonucleotides.

The upstream TSS in human thyroid mRNA was mapped using primer extension with oligonucleotide 16R to a G residue 708 nt 5′ to the initiator methionine and 27 nt 3′ to a TATA sequence (Fig. 3). However, Northern blots of mRNA from thyroid and other tissues showed two distinct hybridizing bands, suggesting that there was a second major TSS or an alternatively processed transcript (Figs. 4 and 5). For example, a coding region probe extending from position 708-1531 hybridized to a doublet of about 6.8 and 7.5 kb in the mRNA obtained from human thyroids and glycoprotein-producing human pituitary tumors 1 and 2, but to only a single 7.5-kb band in placenta and rat BAT mRNA (Fig. 4A). Inspection of the 5′-UTR sequence showed several TATA or TATA-like sequences as well as potential splice/donor and acceptor sites, suggesting the possibility of an alternate TSS or an intron in the 5′-UTR (Fig. 2B). We reprobed the blot with a PCR fragment derived from S2 + 12R (Fig. 2B), corresponding to nucleotides 18–321. This hybridized only to the longest thyroidal mRNA, indicating the presence of an alternative TSS 3′ to these sequences (Fig. 4B). This was confirmed by PCR analyses using as template a 5′-RACE product that had been derived from a RT-PCR reaction primed with oligonucleotide 9R (complementary to nt 1265–1288 in the coding region), poly(C)-tailed, and then amplified with the abridged amplification primer and an antisense primer (oligo 4R in Fig. 2B), subcloned, and sequenced (see *Materials and Methods*). The sequence indicated that the 5′-end of the shorter transcript was nucleotide 676 (Fig. 2B). This TSS is 28 nt 3′ to the TATA-equivalent sequence, AATAA.

To confirm this 3′-TSS, S1 analysis was performed using as primer TSS-2, which is complementary to the coding strand from 666–716. Three specifically hybridizing bands were detected using RNA from human Graves' thyroid tissues, but not in the control RNA from chicken heart tissue (Fig. 5A). The longest band (a) of 56 nucleotides represents a completely hybridized transcript, and bands b and c (~2

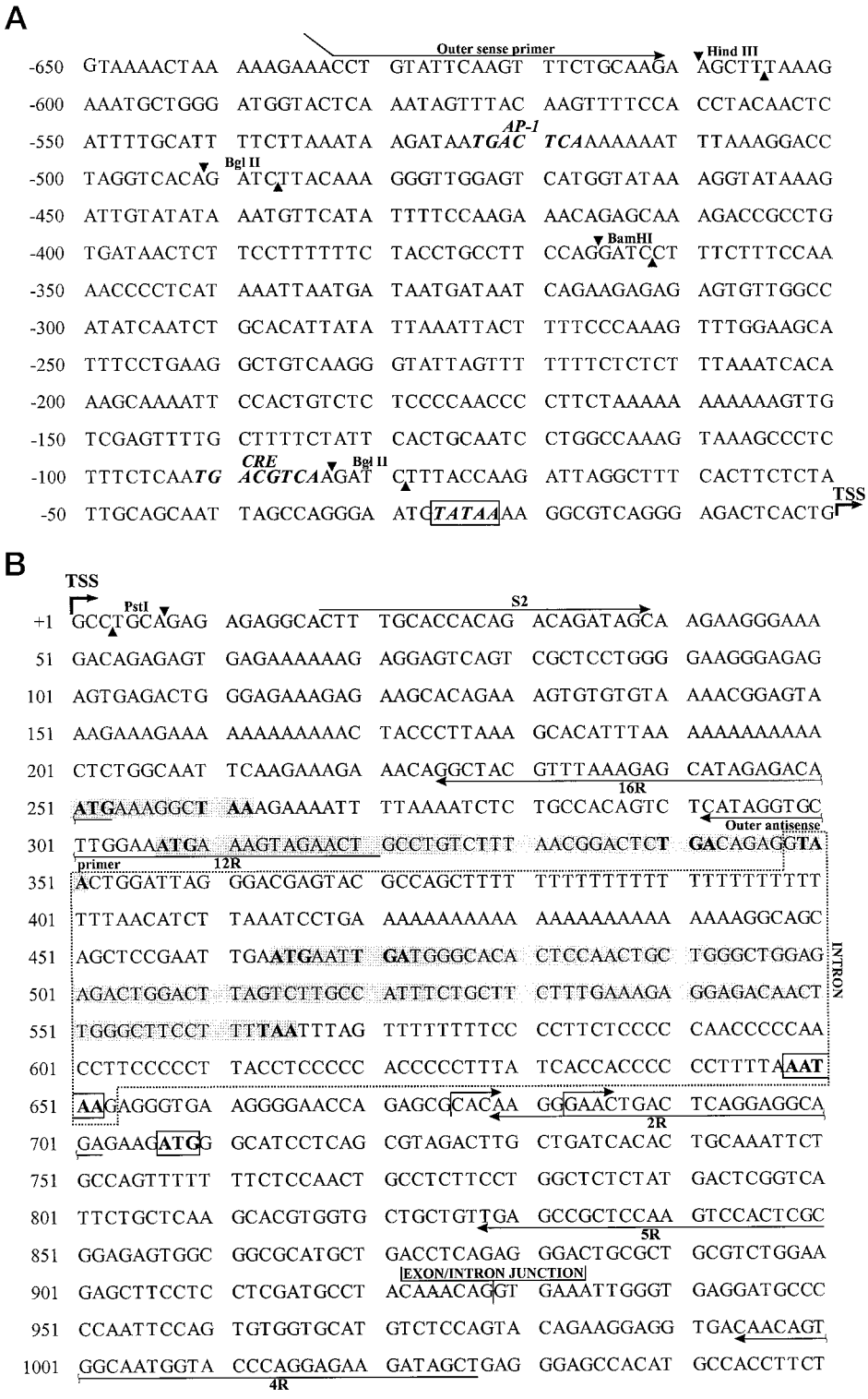

FIG. 2. Nucleotide sequences of the proximal 5′-flanking region (A) and the 5′-UTR and the proximal portion of the coding region (B). The locations of the CRE and AP1 sites are indicated as well as those of various oligonucleotides and critical restriction sites. The alternatively spliced intron is *boxed*, and the short open reading frames in the 5′-UTR region are indicated by shading with the putative initiator methionine and termination codons in *bold*.

and ~35 nt) represent shorter transcripts. The 42-mer corresponds to the transcript defined by the 5′-RACE at 676 nt, 29 nt 3′ to a TATA box equivalent, ATAA. The third TSS was approximately 7 nt 3′ to this. These three bands are found in three different samples of human thyroid RNA with a ratio of about 8.8:1.8:1 by densitometry, suggesting that this is a consistent finding in all thyroid tissues. By Northern analysis, the two shorter transcripts would not be resolved at this position in the gel, and this accounts for the appearance of only two bands. The estimated ratio of the longer to the two shorter transcripts, approximately 3:1, is consistent with the Northern results (Fig. 4A).

To examine the possibility of alternative splicing of the longer transcript, we amplified the product of a RT-PCR

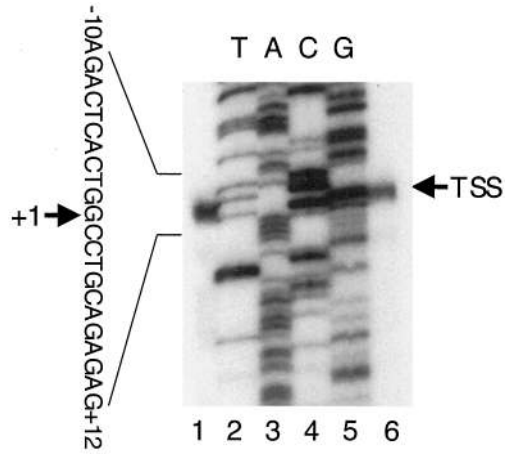

FIG. 3. Primer extension of the hD2 mRNA using primer 16R (nt 225–253; see Fig. 2B). Twenty micrograms of total RNA from human Graves' thyroid tissue were reverse transcribed with ^{32}P end-labeled 16R primer at 42 C for 30 min, and the product was run on a 5% urea-denaturing polyacrylamide gel. Lanes 1 and 6, Primer extension products. Lanes 2–5, Sequencing ladders T, A, C, and G from an h*dio*2 genomic DNA-sequencing reaction using the same primer.

reaction using the sense oligo S2 and the antisense oligo 2R (Fig. 2B). The largest amplicon was about 350 nt, and was subcloned and sequenced. The cDNA was one in which the boxed 306-bp sequence in Fig. 2B was absent. This was confirmed by amplifications using oligonucleotides S2 and 5R, which showed the same 306-nt deletion, suggesting an alternatively spliced intron in the 5′-UTR. Consensus splice donor and acceptor sites are present in the appropriate positions in the sequence (Fig. 2B). Probing the Northern blot with a PCR fragment corresponding to the putative intron showed the same pattern of hybridizing bands as that in Fig. 4B (data not shown), indicating that the putative intron sequences were present only in the longer (7.5-kb) band. However, there was no clear evidence of a transcript approximately 300 nt shorter than the full-length 7.5-kb mRNA on blots probed with either the coding region or nt 18–321 (Fig. 4A). This suggested the possibility that the RT reaction had not faithfully transcribed the 306-bp sequence, perhaps due to tertiary structure in this GC-rich region. Therefore, we performed a repeat RT after preincubation at 65 C for 10 min (see *Materials and Methods*). The product was reamplified as described, but again only the 350-nt band was seen.

For independent confirmation of the presence of an alternatively spliced intron at this position, S1 analysis with a primer complementary to the 3′-boundary region of the putative intron was performed. This confirmed the presence of two transcripts, one containing the intron (band a in Fig. 5B) and another in which the intron was spliced out (band b). The relative densities of bands a and b indicate that most of the D2 transcripts contain the intron despite its exclusion from the PCR-generated cDNAs.

To determine whether the pattern seen in thyroid and pituitary tumor cells was typical of other human tissues, we probed a multi-tissue blot with sequences containing the coding region (Fig. 6A) with nt 18–321 in the 5′-portion of the coding region. The doublet at 7 kb was readily identified in heart and weakly expressed in skeletal muscle (Fig. 6A), but only the shorter band was seen in brain. With longer exposure, the same doublet bands were also seen in kidney and pancreas, but not in liver or lung (Fig. 6A). However, only a single approximately 7.5-kb band was present in placenta, in agreement with the result shown in Fig. 4A. With the 5′-UTR probe, the 7.5-kb band was seen in heart and placenta. In addition, low intensity bands of about 5 kb were seen in all tissues except liver and lung, and less distinct, smaller bands were seen in heart and placenta (Fig. 6B). Probing with the intron fragment showed the 7.5-kb band in heart and placenta, as well as a 2-kb band in pancreas (data not shown). These results indicate that the hD2 coding region is expressed in primary transcripts of 7.5 and 6.8 kb, and that the 6.8-kb mRNA does not contain sequences 5′ to nt 675. A third transcript, the longer mRNA with the intron deleted, is not distinctly identifiable in the Northern blots, except perhaps in brain (Fig. 6A). These three hdio2 mRNA transcripts are depicted schematically in Fig 7.

For the in-frame UGA codon of a selenoprotein to be read as selenocysteine, a SECIS element must be present in the 3′-UTR (17). We have recently identified the SECIS element at the extreme 3′-terminus of the hD2 mRNA, 4.8 kb 3′ to the translation termination signal (3). A probe containing this element hybridizes to both the 7.5- and 6.8-kb transcripts (Fig. 8).

Transcription factor-binding sites in the 5′-flanking region of hdio2

Although computer-assisted analysis of the proximal promoter and 5′-flanking region identified potential binding sites for a number of transcription factors when a single mismatch was allowed, the two canonical sites of greatest interest based on earlier studies in the rat and physiological considerations are the consensus CRE at about −90 and an AP1 site at about −520 (Fig. 2A). An h*dio*2 wild-type (633)CAT plasmid was not induced by a combination of phorbol ester (3×10^{-7} M) and A23187 (10^{-6}-10^{-7} M), suggesting that this site may not be active in the regulation of h*dio*2 gene expression. The h*dio*2 gene constructs are cAMP responsive and discussed subsequently.

The cAMP response element of the hdio2 gene

To confirm the function of the canonical CRE at −92 to −85 bp (Fig. 2A) and to determine whether other CREs are present in the 5′-unsequenced portion of the h*dio*2 gene, the 6.5-kb h*dio*2 5′-flanking region with and without a *Bgl*II deletion and a 633-bp fragment with or without CRE mutations were subcloned into the CAT expression vector pOCAT2 (Fig. 9). Transient expression of h*dio*2 wild-type (633)CAT was increased 3.6-fold by coexpression of the catalytic subunit of PKA (Table 1A). Mutations in the CRE reduced the basal expression of the 633 5′-FR-CAT construct and eliminated the response to PKA, indicating an important role for this CRE in the regulation of expression of this gene. The 6.5-kb h*dio*2 FR-CAT construct was increased about 16-fold by PKA. Deletion of the CRE on a *Bgl*II fragment lowered CAT expression markedly and also eliminated the response to PKA, suggesting that CRE at −90 is the only one present in the h*dio*2 5′-flanking region. To demonstrate that the endogenous h*dio*2 gene responds to cAMP, we prepared pri-

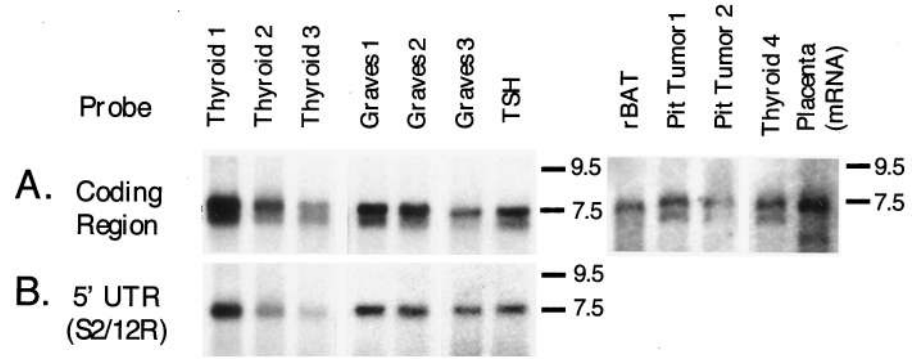

FIG. 4. Northern blot of RNA from human and rat tissues probed with two different portions of the hD2 cDNA. The quantities of RNA electrophoresed were adjusted based on preliminary blots (4) to obtain signals of approximately the same intensity for each lane. A, Blots probed with ^{32}P-labeled cDNA from the coding region of hD2 (nt 708-1531). B, Blot reprobed with ^{32}P-labeled cDNA from the 5′-UTR (nt 18–321). The transcript sizes are indicated in kilobases. Thyroids 1, 2, 3, and 4, Normal human thyroid (20, 20, 20, and 0.5 μg total RNA, respectively); Graves' 1, 2, and 3, human Graves' thyroid (5, 20, and 10 μg total RNA, respectively); TSH, thyroid of a hyperthyroid patient with a TSH-producing pituitary tumor (20 μg total RNA); rBAT, Rat BAT (6.3 μg total RNA); pituitary tumors 1 and 2, α-glycoprotein-producing human pituitary adenomas (0.6 and 2.8 μg total RNA); Placenta, human placenta (2 μg poly(A)$^+$ mRNA). Exposure was for 9 h (A, *left,* and B) and 66 h (A, *right*).

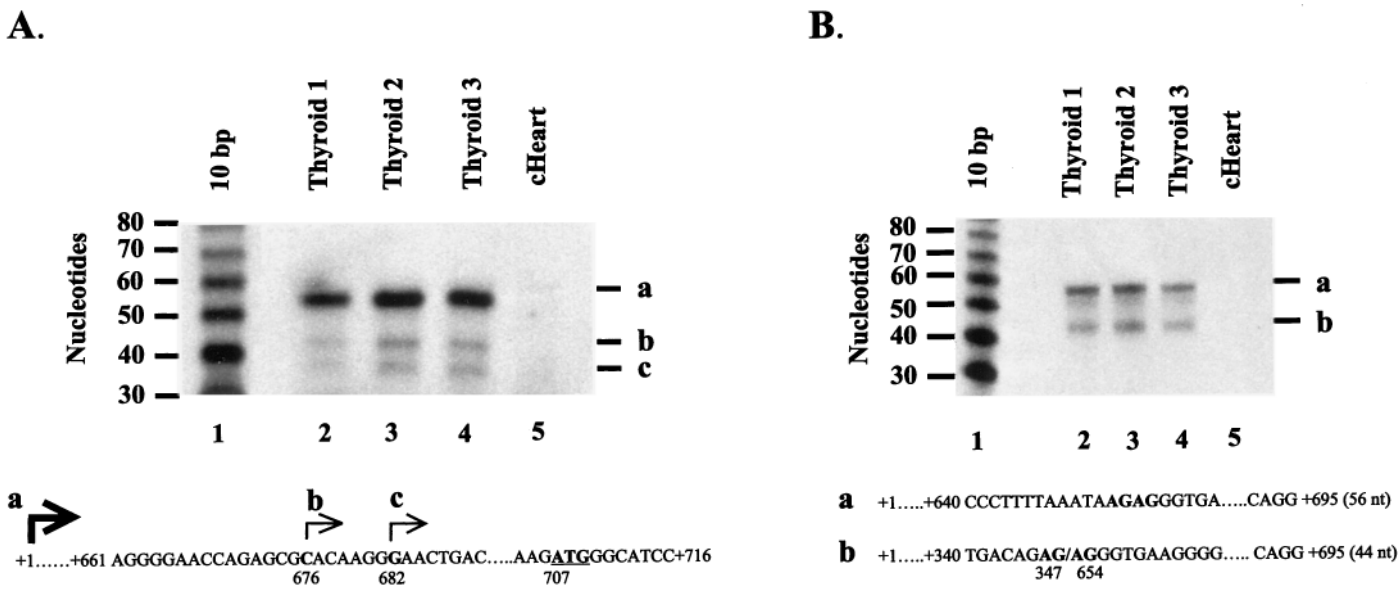

FIG. 5. S1 analysis for the determination of alternative TSS using primer TSS-2 (A) and an alternative splice acceptor site using primer 3′INT (B). 5′-^{32}P end-labeled primer was hybridized with 50 μg total RNA from each tissue, which were subsequently digested by 300 U S1 nuclease at 30 C for 1 h. One half of the reaction product was loaded onto a 6% urea-polyacrylamide denaturing gel. 1, Ten-base pair DNA ladder; 2, 3, and 4, RNA from three human Graves' thyroids; 5, RNA from chicken heart.

mary cultures of human thyroid cells from a functioning human thyroid adenoma. Basal expression of D2 mRNA was barely detectable, but increased more than 10-fold within 3 h of exposure to 10 μM forskolin, confirming the cAMP responsiveness of the endogenous h*dio*2 gene (Fig. 10).

Discussion

Multiple type 2 deiodinase mRNAs are expressed in human tissues

By prolonging the running time of the gel, the broad hD2 mRNA transcript at about 7.5 kb identified in earlier studies by us and others can be resolved into two bands, which differ in size by 500-1000 nt. This doublet D2 mRNA is found in several tissues or cell types, including thyroid, cardiac and skeletal muscle, and α-glycoprotein-producing pituitary tumor cells, and is present in low levels in kidney and pancreas. In this respect, the human D2 mRNA resembles that identified in the rat central nervous system, in which a doublet of about the same size was reported in various brain regions, although not in BAT (1). This same doublet hybridizes to both the coding region sequences and to a SECIS element-containing fragment from the most 3′ 300 nucleotides of the 3′-UTR region (3). In fact, primer extension, PCR and S1 analyses indicate that there are four primary transcripts in thyroid and possibly in several other tissues. These are a full-length 7.5-kb mRNA, the predominant transcript; a 7.2-kb transcript in which an approximately 300-nt intron is spliced out of the full-length transcript; and two shorter transcripts using TSSs just upstream of the ATG directed by a TATA equivalent (AATAA) sequence. All four mRNAs can

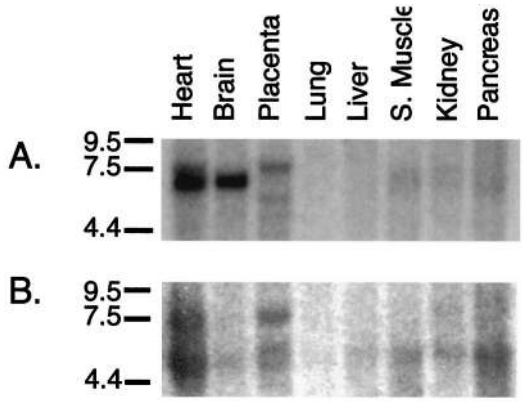

FIG. 6. Northern blot of 2 μg poly(A)$^+$ mRNA from various human tissues. Blot A was hybridized with a coding region probe (see Fig. 4), and blot B was hybridized with the 5′-UTR probe as described for Fig. 4. The transcript sizes are indicated in kilobases.

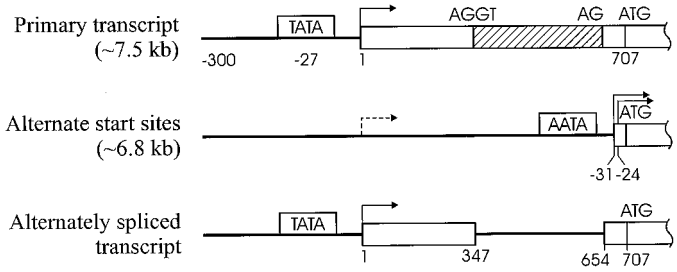

FIG. 7. Schematic diagram of the 5′-regions of the three hD2 mRNA transcripts based on Northern blotting, primer extension and S1 analyses.

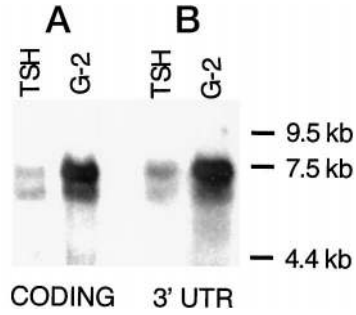

FIG. 8. Northern blot from Graves' (G-2) and TSH-stimulated (TSH) thyroids. Twenty micrograms of total RNA were loaded in each lane. Blot A was hybridized with coding region probe (see Fig. 4). Blot B was hybridized with the SECIS-containing cDNA fragment constituting the terminal 300 bp of the 3′-UTR (excluding the poly(A)$^+$ sequences) (3).

encode a full-length D2 selenoprotein. Expression of D2 mRNA in human kidney and pancreas has not been reported previously. We also noted that 3′-fragments of the D2 cDNA containing the SECIS element are found in expressed sequence tag sequences from libraries prepared from prostate, breast, and uterus, which have also not previously been shown to express D2 mRNA or activity (3). Interestingly, placenta is the only human tissue in which only the longer of the two major transcripts is expressed. However, in the Northern blot shown in Fig. 6A, the longest brain mRNA is shorter than that in placenta but longer than 6.7 kb, suggesting that it represents the 7.5-kb transcript with the intron

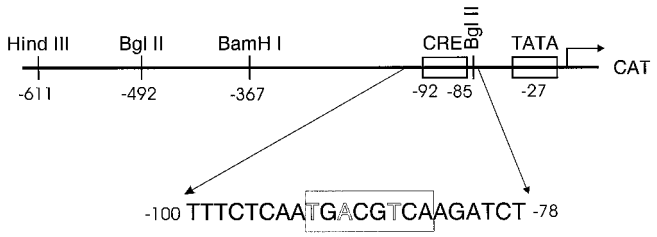

FIG. 9. Diagram of h*dio*2 mut CRE CAT showing the changes introduced in the consensus CRE. The mutated sequence was AGTCGACA.

TABLE 1. Mutation or deletion of the CRE at −90 eliminates the response of the h*dio*2 gene to protein kinase A (PKA) α-catalytic subunit when transiently expressed in HEK-293 cells

Construct	n	Plasmid cotransfected (0.1 μg)	
		Vector	αPKA
A. CRE mutation			
1. h*dio*2 wild-type 633 CAT	6	1.04 ± 0.19	3.62 ± 0.42[a]
2. h*dio*2 mut CRE 633 CAT	6	0.16 ± 0.03	0.10 ± 0.01[b]
B. CRE deletion			
1. 6.5 h*dio*2 CAT	6	0.48 ± 0.05	7.8 ± 0.7[a]
2. 6.5 h*dio*2 ΔBglII CAT	6	0.012 ± 0.005	0.027 ± 0.001[b]

All results are the mean ± SD of cellular CAT/medium hGH in six plates of HEK cells transfected with 100 ng of a plasmid expressing the α-catalytic subunit of PKA or an empty vector.

[a] Significantly different from Vector by ANOVA followed by Newman-Keuls ($P < 0.001$).

[b] Significantly different from wild-type by ANOVA followed by Newman-Keuls ($P < 0.001$).

deleted. The presence of a potential intron in this same general location was suggested by Celi *et al.*, although no sequence information was provided (18).

Although the 7.5-kb transcript is the most highly expressed in human thyroid, there is no systematic variation in the ratio of longer to shorter transcript based on the type of thyroid abnormality. It might be expected that thyroid from Graves' patients or the TSH-stimulated thyroid would have a higher ratio of the 7.5-kb to the 6.8-kb species if the CRE has a greater effect on transcription from the 5′- than from the 3′-TSS. This does not seem to be the case. The reason(s) for this is not obvious, but could relate to differences in the half-lives of the two species, recalling that the specimens were all obtained under the constraints of surgical procedures and that the half-life of rD2 mRNA is short (~2 h) (19). Furthermore, the levels of thyroid-stimulating Ig were not measured in the Graves' patients and may vary considerably. There are marked variations in D2 activity between Graves' thyroids, suggesting considerable heterogeneity in this population (see below) (4).

The 5′-UTR of the hD2 mRNA

The previously reported partial hD2 cDNA clone Z44085 has its 5′-terminus at nucleotide +553 (within the alternatively spliced intron) (1, 2). The rat D2 5′-UTR (accession no. AB011068) is 74% identical to the h*dio*2 cDNA between nt 437 and the ATG (1). Another 257-nt fragment of the rat D2 partial cDNA (nt 1–257) has 73% identity to nt 152–406 of the human mRNA. This fragment includes an alternative splice donor sequence GG/GT in the identical location as the hD2

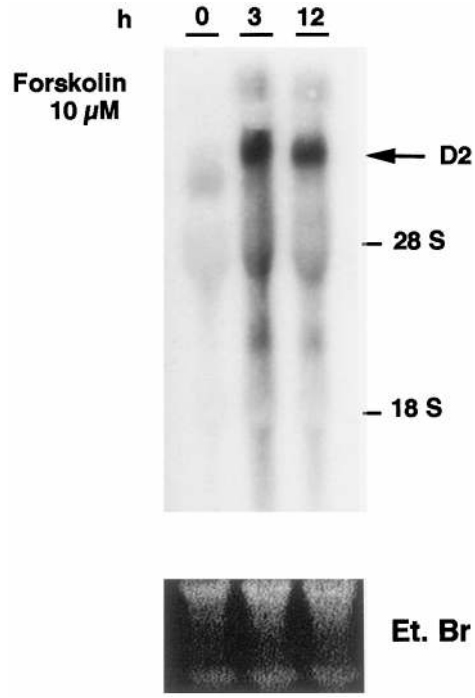

FIG. 10. Northern blot of 30 μg total RNA from dispersed human thyroid cells before and after exposure for 3 and 12 h to forskolin (10 μM). Thyroid cells from a functioning human adenoma were dispersed by collagenase, seeded in petri dishes, and cultured in Ham's F-12 medium supplemented with 10% FCS. After harvest, electrophoresis, and capillary transfer, the blot was probed with a ^{32}P-labeled coding region hD2 probe (nt 708-1531) at high stringency, washed, and exposed for 72 h. Ethidium bromide staining was used to ensure equal loading and transfer.

AG/GT (nt 347–350), raising the possibility of a similar, alternatively spliced intron in the rat *dio*2 gene. This or an alternative TSS, such as observed in the human gene, could explain the doublet rD2 mRNA band in the Northern blots from the central nervous system reported previously (1). Recently, the sequence of a portion of the human chromosome 14q24.3 containing the complete sequence of the human *dio*2 gene has appeared in GenBank (AC007372) and agrees with that in Fig. 2. Aside from this, the only sequence in the GenBank database with significant (59%) similarity to sequences 5′ to nt 152 is the recently reported chicken D2 cDNA (AF 125575) (20). Interestingly, the splice donor site is also conserved in that cDNA at the same position. However, only a single D2 transcript is found in chicken Northern analyses (20).

Two short (3- and 4-codon) and one long (12- or 15-codon) open reading frames (ORFs) are present in the 5′-UTR. Interestingly, the position and length of the first two ORFs are conserved in the rat *dio*2 5′-UTR, with the amino acid sequence of the larger putative peptide starting at nt 307 (MKVELPVFNGL-UGA-QR-UAA) virtually identical to one in the rat 5′-UTR starting at nt 129. A potential ORF of 33 codons starting with the AUG at nt 464–6 is presumably not initiated, as the Kozak sequence is quite poor. The first two ORFs have favorable Kozak sequences, a purine at −3 in the absence of a G at +4, whereas the putative initiator methionine of the major ORF of hD2 has both the −3 purine and a G residue at +4 (21). In about 90% of eukaryotic mRNAs, the most 5′-AUG is used as the transcription initiation site. However, the exceptions to this rule, *i.e.* mRNAs that contain one or more upstream ORFs, encode a group of proteins with interesting functions, such as protooncogenes, transcription factors, DNA-binding proteins, and receptors for various hormones, including thyroid, estrogen, and retinoic acid (reviewed in Refs. 21 and 22). In general, such AUG-burdened 5′-UTR sequences impair translation, suggesting that these encode proteins for which excessive production has adverse consequences (21). How such mRNAs are translated has not been resolved despite considerable investigation. Two models are proposed. One is the yeast GCN4 mRNA model, in which ribosomes can reinitiate at downstream codons depending on the distance between the upstream and downstream ORFs and the concentration of the initiator ternary complex (reviewed in Refs. 23 and 24). The second model is that of the internal ribosomal entry site, as exemplified in the picornaviruses (reviewed in Ref. 22). Given the conservation of position and sequence of the sORFs between human and rat, it seems likely that the D2 mRNAs will follow the GCN4 model, but there are no well studied mammalian examples of this.

Of special interest for the SECIS element-containing D2 mRNAs is the fact that there is an in-frame UGA in the second sORF (although followed by an in-frame UAA 3 codons 3′ when the intron is present) and that the third sORF terminates with UGA. There is an in-frame UAA for this ORF at nt 564–566, but the deduced protein from this ORF would be 33 residues in length. A similar phenomenon would occur in the rat mRNA with a highly similar deduced peptide (73% identity). Whether the SECIS element would suppress the stop codon function in these 5′-UTRs at this distance from the SECIS element is not clear. It has been shown that the choice between a UGA stop and a UGA selenocysteine codon is strongly influenced by the nucleotide following the UGA (the fourth base) (25). A purine in this position favors termination, whereas pyrimidine residues, present as the fourth base for each of the UGA codons in the hD2 5′-UTR as well as the UGA in the highly conserved catalytic center, favor selenocysteine insertion (25). Interestingly, the 3′-UGA in the carboxyl-terminus of D2 is followed by a purine consistent with its frequent function as a stop codon, as we have seen in transient expression assays (2). Further studies will be required to unravel this issue, which is made more intriguing by the fact that if the 3′-TSSs are used, none of the sORFs is included. Nontheless, human placenta and rat BAT express only the longer transcript, and D2 activity is clearly expressed in these tissues (26, 27).

The CRE and other transcription factor-binding sites in the hD2 promoter and 5′-flanking region

Analysis of the proximal 5′-flanking region indicates consensus binding sites for two ligand-dependent transcription factors previously reported to increase D2 activity in BAT or in rat glial cells, a CRE and an AP1 site (9, 28, 29). Despite the presence of a consensus AP1-binding site, there is no response of the 633-bp h*dio*2 CAT construct to phorbol esters with or without calcium ionophore. On the other hand, the CRE confers a potent response to the 5′-TSS and is located in

a typical position within 100 nt of the upstream TATA box. Mutation or deletion of the CRE eliminates the cAMP response of the h*dio*2 gene. The lack of a significant residual response of the *Bgl*II-deleted construct suggests that it is the only CRE in the 6.5-kb 5′-flanking region. A response to cAMP of the h*dio*2 promoter is consistent with the high D2 mRNA expression of some human Graves' or TSH-stimulated thyroid relative to that of normal glands (4). We found D2 activity levels 30–100 times higher than normal in two such thyroids (4). Furthermore, the presence of high levels of D2 mRNA in patients with toxic adenomas due to presumptive activating mutations in the TSH receptor or G_s subunit could also be explained by this CRE (30). Forskolin increased the D2 mRNA over 10-fold in human thyroid cells in culture, establishing that the high expression of D2 mRNA in Graves' and TSH-stimulated thyroid tissues can be cAMP mediated. Recently, TSH and/or cAMP were shown to increase D2 activity in cultured human thyroid cells as well (31, 32).

In summary, the present results demonstrate a complex pattern of expression of the h*dio*2 gene in human thyroid and other tissues. The presence of a relatively long 5′-UTR with several sORFs suggests that translation of this mRNA is carefully modulated through as yet unclear mechanisms common to a small fraction of mammalian genes. However, use of the downstream TSSs would bypass these ORFs, possibly enhancing translation. These sORFs together with the recent results showing that rat D2 has a short half-life and is degraded in proteasomes suggest that the level of this enzyme is tightly controlled at both transcriptional and post-transcriptional levels (33). The presence of a potent CRE is consistent with the brisk physiological response of this promoter to cAMP. The availability of the 5′-FR of this gene and the mapping of the 5′-UTR will allow study of the mechanisms involved in the tissue-specific expression of this protein as well as the determination of whether its transcription is negatively regulated by T_3 as is the rat *dio*2 gene (19). The CRE-containing h*dio*2 promoter could be another example of a promoter for which competition for CRE-binding protein and other basal transcription factors between T_3-thyroid hormone receptor complex and the CRE-CRE-binding protein complex would cause negative regulation by T_3 (34).

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