The mRNA Structure Has Potent Regulatory Effects on Type 2 Iodothyronine Deiodinase Expression

BALÁZS GEREbens, ANNA KOLLár, JOHN W. HARNEY, AND P. REED LARSEN

Institute of Experimental Medicine (B.G.), Department of Neurobiology, Budapest H-1083, and University of Pecs, Faculty of Sciences, Institute of Biology, Pecs H-7624, Hungary; Szent István University (A.K.), Faculty of Veterinary Science, Department of Physiology and Biochemistry, Budapest H-1078, Hungary; Thyroid Division (B.G., J.W.H., P.R.L.), Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Type 2 deiodinase (D2) is a selenoenzyme catalyzing the activation of T4 to T3. D2 activity/mRNA ratios are often low, suggesting that there is significant posttranscriptional regulation. The D2 mRNA in higher vertebrates is more than 6 kb, containing long 5’ and 3’ untranslated regions (UTRs). The D2 5’UTRs are greater than 600 nucleotides and contain 3–5 short open reading frames. These full-length 5’UTRs reduce the D2 translation efficiency approximately 5-fold. The inhibition by human D2 5’UTR is localized to a region containing the first short open reading frame encoding a tripeptide—MKG. This inhibition was abolished by mutating the AUG start codon and weakened by modification of the essential purine of the Kozak consensus. Deletion of the 3.7-kb 3’UTR of the chicken D2 mRNA increased D2 activity approximately 3.8-fold due to an increase in D2 mRNA half-life. In addition, alternatively spliced D2 mRNAs but not encoding an active enzyme are present in both human and chicken tissues. Our results indicate that a number of factors reduce the D2 protein levels. These mechanisms, together with the short half-life of the protein, ensure limited expression of this key regulator of T4 activation. (Molecular Endocrinology 16: 1667–1679, 2002)

Type 2 deiodinase (D2) catalyzes the 5’ monodeiodination of T4 to T3, the first step found in the process by which T4 produces its effects. It is widely expressed in humans being in brain, pituitary, brown adipose tissue, placenta, skeletal, and cardiac muscle and skin (1, 2). Because D2 contains the rare amino acid selenocysteine encoded by UGA in its active center, its success in translation requires specific sequences, the selenocysteine insertion sequence (SECIS) element, in its 3’ untranslated region (UTR) (3). Even so, its translational efficiency, like that of other selenoproteins, is much less than that of typical proteins (4). The process can also be influenced by selenium and by the components of the selenocysteine insertion machinery (5–7).

Interestingly, D2 activity/mRNA ratios show marked variations. In tissues such as the normal human thyroid, D2 activity is lower than would be expected from the amount of D2 mRNA expressed. For example, in the thyroid tissue of a Graves’ patient, the D2 activity/mRNA ratio was 25-fold that in normal human thyroid (8, 9). In human thyroid adenomas, the D2 activity/mRNA ratio is also higher than in normal thyroid, indicating that there are variations in posttranslational processing (10).

It is not understood which components of the highly complex D2 regulation might be involved in the generation of these variations in mRNA/activity ratios between tissues. The dio2 (type 2 iodothyronine deiodinase) gene has multiple transcription start sites (TSSs), and the transcriptional regulatory component involves cAMP, thyroid transcription factor-1, TSH, and activator protein-1 regulated pathways (11–15). In addition, TSH or 3,3’5’ triiodothyronine (reverse T3) cause significant posttranslational down-regulation. The latter effect involves substrate-accelerated selective proteolysis via the ubiquitin/26 S proteasome pathway (16–18).

The D2 mRNAs of vertebrates are unusually long (~6–7 kb) compared with the 2- to 2.5-kb length of the other two members of the selenodeiodinase family. Despite this large size, the open reading frame (ORF) encoding the active enzyme is only approximately 800 bp. The 3’UTR is extremely long (~4.7 kb) and contains a SECIS element at its 3’ end. This is the greatest distance yet recognized between a SECIS element and the UGA codon and could theoretically attenuate successful readthrough of the two UGA codons in the mRNA (19–21). The 5’UTR of the D2 mRNAs is at least approximately 600-bp long and the mRNAs of the four species cloned all contain 3–5 short ORFs (sORF), which may also modulate translation. The goal of our study was to elucidate the role of mRNA structure in the posttranscriptional regulation of this key rate-limiting gene in thyroid hormone activation.

Abbreviations: c, Chicken; D2, type 2 deiodinase; dio2, type 2 iodothyronine deiodinase gene; DTT, dithiothreitol; h, human; hD2, human D2; HEK, human embryonic kidney; hGH, human GH; mut, mutant; nt, nucleotide; ORF, open reading frame; rT3, reverse T3; SECIS, selenocysteine insertion sequence; sORF, short ORF; TSS, transcription start site; UTR, untranslated region; wt, wild-type.
RESULTS

The Translation of D2 mRNA Is Regulated by Its 5’UTR

Comparison of the 600- to 700-bp 5’UTR of the dio2 genes of higher vertebrates shows a number of common features. The human D2 (hD2) 5’UTR has 73, 70, and 46% identity to the rat, mouse, and chicken sequences, respectively. Virtual identity exists in the most 5’ approximately 20 bases, which form putative stem loop structures by GCG FOLDERNA analysis (Fig. 1A). The more 3’ portions of these 5’UTR contain 3–5 putative sORFs (Fig. 1B). The sORFs were defined considering the unique feature of selenoprotein mRNAs in which SECIS elements in the 3’UTR suppress the stop codon function of UGA codons. Unambiguous stop codons (UAA and UAG) and in-frame UGA codons followed by purines were considered as translational terminators, whereas in-frame UGAs followed by pyrimidines favor readthrough and are indicated by an asterisk (22). The deduced peptide sequences of these ORFs have low similarity between species with the exception of rat and mouse (Fig. 1B).

We developed a chicken D2 (cD2)-containing reporter system to explore the potential role of these 5’UTR sequences in modulating the translation efficiency of this selenoprotein. The human and chicken cD2 5’UTR caused a 5-fold reduction in transient cD2 expression in HEK-293 cells (Fig. 2). This was not due to the conserved sequences in the 5’ region (Fig. 1A) because deletion of these had no effect. The 5’UTR of cD2 was as effective as that of the human mRNA in decreasing D2 activity. Northern blots showed no inhibitory effect of the sORFs on the transient expression of the cD2 mRNA (data not shown). In fact, the D2/actin mRNA/human GH (hGH) ratio of full-length cD2 was about 3-fold higher than that of the Δ5’UTRcD2 construct, whereas the activity in the same experiments was 2- to 3-fold lower. The effect on D2 activity of 5’UTR fragments containing the hORF-A was not different from that of the hD2–5’UTR containing the sORFs. However, constructs lacking the sORF-A were significantly less effective in sup-

Fig. 1. Alignment of D2 5’UTR Portions

A, Alignment of the 5’ portion of the human, chicken, rat, and mouse D2 mRNAs. The region just 3’ to the transcription start site is highly conserved. Alignments were performed by GCG PILEUP. B, Deduced amino acid sequences of the putative amino acid sequence of the sORFs in the human, chicken, rat, and mouse D2 5’UTR. Unambiguous stop codons and in-frame UGAs followed by purines were considered as translational terminators. In-frame UGAs in possible readthrough position (codon followed by a pyrimidine) are indicated by an asterisk (22). The presence of a strong translational initiation sequence (−3 purine where position 1 is the A of the start codon) is indicated by a plus sign (39).
pressing D2 activity than those containing sORF-B or C (Fig. 3). Thus, the sORF-A encoding the tripeptide MKG was the predominant translation inhibitory region in the hD2 5′UTR.

Mutagenesis was used to inactivate the AUG codon of the human sORF-A and the shortest chicken ORF-B to determine the structure-activity relationships (Fig. 1B). Replacement of ATG by TTG completely abolished the inhibitory effect of the sORFs on D2 translation (Fig. 4). The suppressive activity of human sORF-A was also decreased by changing the Kozak consensus replacing the −3A with C. These data in-
The sORF based translational inhibition of D2 activity is not restricted to cell types not expressing endogenous D2.

**Table 1. Translational Inhibition of D2 by hORF-A in Different Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>hORF-A-(Wt)-cD2</th>
<th>hORF-A-(Mut-ATG)-cD2</th>
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<tbody>
<tr>
<td>GH4C1</td>
<td>54 ± 0.05</td>
<td>96 ± 11*a</td>
</tr>
<tr>
<td>Met5A</td>
<td>43 ± 1.8</td>
<td>68 ± 5.3*a</td>
</tr>
<tr>
<td>COS-7</td>
<td>39 ± 0.8</td>
<td>92 ± 5.7*a</td>
</tr>
<tr>
<td>JEG</td>
<td>50 ± 6.1</td>
<td>85 ± 12*a</td>
</tr>
</tbody>
</table>

*a P < 0.05 vs. hORF-A-(Wt)-cD2 of the corresponding cell line by unpaired t test. Experiments were performed in duplicate and repeated at least three times.

surrounded by adenine/uracil (A/U) rich regions as is characteristic of instability motifs (19, 21, 23).

The cD2 3′UTR was restricted to remove 3710 nucleotides (nt) containing 8 of the 9 AUUUA motifs between the coding region and the SECIS element (cD2–3.7Δ, in Fig. 6A). To keep the distance constant between the active center and the SECIS element, exogenous DNA from *Neurospora crassa* or from the mouse thyroid receptor/Δ251 without AUUUA motifs was inserted (cD2–3.7exo). Replacement of the 3′UTR increased D2 activity by approximately 50%, whereas elimination of the 3′UTR increased D2 activity approximately 3.8-fold relative to the cD2wt (Fig. 6A). Transfections were performed with 1, as opposed to 10, 3D2-expression plasmid with the same result, indicating that the selenoprotein synthesis was not saturated.

The 3′UTR of the vertebrate D2 mRNAs is at least 4.7 kb, and each contains the required SECIS element at its 3′ terminus (19–21). The human, chicken, and mouse D2 3′UTR (GenBank accession no. AF096875) also contain 11, 9, and 7 AUUUA putative mRNA instability motifs, respectively. These sequences are
To evaluate possible differences in mRNA stability, similarly transfected cells were exposed to tetracycline to stop transcription from the tetracycline-repressible promoter (see Materials and Methods). After 13 h of tetracycline exposure, the amount of cD2–3.7exo mRNA was approximately 2.5-fold higher than in the cells transfected with the cD2wt (Fig. 6B). This suggested that elimination of the 8 AUUUAs contained in the 3’UT (cD2–3.7) increased the mRNA half-life. As is apparent in Fig. 6B, deletion of most of the 3’UT (cD2–3.7Δ) leads to the presence of large quantities of two D2 transcripts. The larger transcript is of the expected size (approximately 2.1 kb). The blot was reprobed using a cD2 SECIS region probe, and the same doublet was observed indicating that both mRNAs are potentially functional. The densities of the bands were 15-fold (larger) and 40-fold higher than the wt, respectively.

Cloning and Functional Testing of Alternatively Spliced D2 Coding Region Transcripts in Chicken and Human Tissues

Despite similar D2 mRNA levels in the telencephalon and liver of the adult chicken (21), the maximum velocity for D2 is 2.6-fold higher in brain tissue (408 vs. 156 fmol T4/h·mg protein). While only a single 6.1-kb D2 was observed in both tissues, a potential explanation for this discrepancy is the presence of a second mRNA in liver similar in size but not encoding an active protein. This proved to be the case with cDNAs cloned from two animals demonstrating a 77-bp deletion just 5’ to the region corresponding to the exon/intron junction in the human D2 coding region (Fig. 7A). The absence of these nucleotides resets the reading frame and the deduced amino acid sequence of the D77cD2 protein is terminated short of the active center (Fig. 7B). The coexistence of the normal and D77cD2 mRNA was shown in the telencephalon and liver by PCR (Fig. 7C). As predicted, the D77cD2 mRNA did not encode an active D2 in transiently transfected HEK-293 cells.

Alternatively spliced human D2 mRNAs involving the same intron/exon junction were also found in human thyroid tissue samples (see Materials and Methods). Three samples had two transcripts (see Fig. 8A). The cDNAs of the three abnormal (longer) transcripts were cloned and sequenced. All had an identical 108-bp in-frame insertion that also contained an in frame TGA followed by a C, a context that can support UGA translation (Fig. 8B). To see if this mRNA could encode a functional D2, the hD2+108 coding region was placed 5’ to a SeIP SECIS element with a FLAG epitope fused to the NH2 terminus. The protein was transiently expressed in HEK-293 cells in Na2(75Se)O3 containing media and immunoprecipitated by anti-FLAG antibody. Autoradiography showed that the hD2+108 coding region encoded a protein of approximately 35 kDa (Fig. 8C). This corresponds to the calculated size based on the deduced amino acid sequence of the putative full-length D2+108 protein indicating that the UGA is translated. No band of this size was visible in the control lane. The hD2+108 transfected HEK-293 cell sonicates contained no D2 activity. Sequence analysis showed that the 108 bp is derived from the midportion of the approximately 8-kb intron separating hdiø2 exon α and β (Fig. 8D).

DISCUSSION

Our results document several previously unrecognized characteristics common to the D2 mRNAs of four dif-
ferent species which can explain discrepancies between D2 mRNA content and D2 activity in different tissues. Quantitatively most important is the effect of the 5'UTR, which causes a 5-fold reduction in D2 expression in HEK-293 cells (Fig. 2). While there are 3–5 sORFs in the 5'UTRs of the human, chicken, rat, and mouse D2 mRNAs (Fig. 1B), in the human D2 it is the most 5' sORF (hORF-A), which is primarily responsible for this effect (Fig. 3). These sORFs encode a tri- (human, rat, mouse) or di-peptide (chicken). The hORF-A operates only in cis and requires an AUG codon. All of the shortest D2 5'UTR ORFs have Kozak consensus sequences with purines at −3 and +4 relative to the ATG, and an A to C substitution at −3 of the hORF-A reduces the translational inhibition by about 2-fold (Fig. 4). The inhibitory effect was not D2-specific (Fig. 5). Our results suggest there is no tissue specificity to the inhibitory function of hORF-A (Table 1).

The best characterized examples of sORF-based translational regulation occur in yeast and viruses. The translation of the yeast GCN4 transcription factor is increased by amino acid starvation. This process is regulated by the 4 sORFs in the 5'UTR in a manner independent of the amino acid sequence of the putative peptides. The phosphorylation state of eIF-2 controls the re-initiation frequency at ORF4 (see Ref. 24 for review). In contrast, the yeast CP1 leader expresses a 25 amino acid leader peptide that suppresses the translation of the glutaminase subunit of the carbamoyl-phosphate synthetase in a sequence-specific manner (25). Another well-documented mechanism for bypassing sORFs occurs in Picornaviruses where specific cis acting RNA sequences, the internal ribosome entry segment, allow translation of major 3' ORFs (26). On the other hand, the Cauliflower mosaic virus 35S RNA utilizes nonlinear ribosome migration, ribosome shunting, to bypass the inhibitory sORFs (for review see Ref. 27).

Fig. 6. Effect of the cD2 3'UTR on mRNA Levels and D2 Activity

A. Expression constructs containing cD2 with intact (cD2wt), replaced (cD2–3.7exo) or deleted (cD2–3.7Δ) 3'UTR were prepared. A non-AUUUA containing fragment of 3.7 kb originating from the Pho2 gene of Neurospora crassa and the mouse TRα was inserted into the cDNA (see Materials and Methods) and transiently transfected into HEK-293 cells. D2 activity was measured in duplicate HEK-293 plates transfected with cD2wt, cD2–3.7exo and cD2–3.7Δ constructs in at least three separate experiments. Data are the mean ± SEM % D2 activity normalized for transfection efficiency for hGH (cpm/100 μl media). *, P < 0.01 vs. cD2wt by ANOVA followed by Newman-Keuls. B. The amount of D2 mRNA quantitated by Northern blotting in two separate experiments 13 h after inhibition of transcription using 1 μg/ml tetracycline as described in Materials and Methods. Both of the doublet bands in cD2–3.7Δ transfected cells hybridized with both cD2 coding- and SECIS-containing sequences.
The translational mechanism for higher vertebrates containing a 5′UTR with sORFs upstream of the major coding sequence is poorly understood. Only 10% of 5′UTR vertebrate mRNAs contain such sequences (28). This group of genes includes transcription and growth factors, proto-oncogenes, receptors of the G protein-coupled receptor superfamily, and signal transduction components (29). Examples include Angiotensin II type IA receptor (30), CCAAT/enhancer-binding (31), ornithine decarboxylase (32), fibroblast growth factor-5 (33), the alcohol dehydrogenase-5/formaldehyde dehydrogenase and Myf6 genes (34), growth factor-5 (33), the alcohol dehydrogenase-5/giotensin II type IA receptor (30), CCAAT/enhancer-transduction components (29). Examples include An-protein-coupled receptor superfamily, and signal growth factors, proto-oncogenes, receptors of the G (28). This group of genes includes transcription and 5′ coding sequence is poorly understood. Only 10% of the human D2 sORF-B also has a sequence but common to all D2 mRNAs. Interestingly, the suppressive effect is independent of peptide sequence and 3′UTRs are not conserved between species, not even between the most potent sORF-A, indicating that the suppressive effect is independent of peptide sequence but common to all D2 mRNAs. Interestingly, the human D2 sORF-B also has a 3′ purine (G), but this sORF does not suppress D2 translation (Figs. 1B and 3). This, taken together with the successful initiation from the weak translation initiation sequence of the β2 adrenergic receptor sORF, suggests in accordance with the findings of Kozak (39) that other factors, such as secondary structure, should be taken into consideration when assessing the translational inhibitory potential of a 5′UTR sORF. The 5′ terminal end of the D2 5′UTRs have high sequence similarity (Fig. 1A) and computer-assisted methods predict secondary structures for this region. However, we found no suppressive effect of this portion of the human D2 5′UTR on D2 translation.

It is not yet clear whether the translational suppression caused by the D2 5′UTR is constitutive or inducible. Within a given cell type or tissue, increases in D2 mRNA, such as those induced by adrenergic mechanisms in pineal gland or during development, increase D2 activity more or less in parallel (40–42). However, recent analyses of D2 activities in hyperfunctioning human thyroid adenomas show that the ratios between adenoma and normal are greater for D2 activity (approximately 5-fold) than for D2 mRNA (approximately 3-fold), suggesting there may be more efficient translation as well as increased mRNA as an explanation for increased D2 (10). Further studies will be required to explore these possibilities.

The long 3′UTR of the D2 mRNA has many AUUUA instability motifs and the D2 mRNA has an approximately 2-hour half-life (14, 19, 21). The study of the functional role of these structures is complicated by the fact that reducing the length of the 3′UTR simultaneously alters the relative proximity of the SECIS element to the UGA in the catalytic center of the coding region. Increasing the distance between the SECIS element and the coding region of the rat type 1 deiodinase by 1.5 kb does not decrease D1 expression (43). However, the larger change of 3.7 kb could have such effects. Substitution of Neurospora and mouse thyroid receptor α DNA, which have no AUUUA elements, for the chicken D2 3′UT cause a 50% increase in transient D2 expression (Fig. 6A). However, the amount of D2 mRNA 13 h after inhibition of D2 transcription was 2.5-fold higher (Fig. 6A). Because both the cD2wt, cD2–3.7exo, and cD2–3.7Δ mRNAs were expressed using the same promoter contained in the same nucleotide context, this finding suggests that the half-life of the chimeric mRNA was prolonged. Deletion of the 3.7-kb fragment increases D2 activity 3.5-fold, but there is a much greater increase in the mRNA after blocking transcription, again suggesting that the truncated mRNA is more stable.

Even though the structures of the transcripts in cD2–3.7Δ transfected cells are not defined (Fig. 6B), both the shorter and longer mRNAs hybridize with both coding sequence and the SECIS element containing probes indicating that both can potentially be translated. The differences in D2 expression are not changed by a 10-fold reduction in transfected plasmid making it highly unlikely that saturation of one or more of the components required for selenoprotein synthesis limits translation. Thus, we are unable to address definitively the role of the 3′UTR in D2 translation efficiency.

**Alternative Splicing in the Coding Region of the D2 mRNA Gives Rise to Inactive D2 Proteins**

At least three 6- to 7-kb D2 mRNA transcripts differing by 500–700 nt have been identified in human thyroid, and two in brain and other tissues (1, 9, 11). However, differences of 50–100 nt are impossible to recognize by size alone in such a long mRNA. We have identified two D2 transcripts in chicken brain and liver, one of which encodes an inactive protein. The cDNA encoding the inactive D2 lacks 77 nt in the coding region, apparently due to the use of an alternative splice site. The genomic organization of the cdio2 gene is not known but the 3′ end of the missing cD2 77 bps maps exactly to the position of the exon/intron junction of the mouse and human dio2 genes, suggesting the alternative processing of an intron in the same position (Fig. 7A) (11, 20, 44). PCR studies show that both wt and Δ77cD2 transcripts are expressed in both telencephalon and liver and different ratios could account for different D2 activity/mRNA ratios.
Fig. 7. Sequence and Expression of the Δ77cD2 Transcript

A. Alignment of the coding region of the chicken and human D2 coding regions (GenBank accession nos. AF125575 and U53506, respectively) to the sequence of the cD2. Seventy-seven base pairs (underlined) are missing from the cD2-clone no. 2. 5' and 3' nucleotides of the spliced region are indicated in bold. A polymorphism of cD2-clone no. 1 is shown in italics. The insertion site of the hdoi2 intron is marked by an arrow. B. Nucleic acid and deduced amino acid sequences of the alternatively spliced cD2 mRNA. The wt cD2 amino acid sequence is underlined and bold, the new junction is underlined and shown in bold italics. Numbers show positions in the cD2 mRNA GenBank accession no. AF125575. C. Agarose gel electrophoresis of the PCR products from wt cD2 mRNA from telencephalon (T) and liver (L) from an adult chicken using Bp3 and Bp90 oligos (sequences are indicated in Table 2). The expected 277-bp band and a second approximately 200-bp product were generated. The products
Similarly, an alternatively spliced hD2 mRNA encoding an inactive protein is expressed in human thyroid tissue. This transcript is readily seen on RT-PCR using oligonucleotides spanning the exon-intron junction between the two coding exons α and β (Fig. 8, A and D). It derives from an alternative 5’ exon-intron junction leading to insertion of 108 intronic nucleotides after codon 74 (Fig. 8, B and D). The insertion does not change the reading frame and is translated even though it contains an additional UGA codon (Fig. 8, B and C). It has no catalytic activity. Parallel with the cloning of the thyroidal hD2 5’-108 coding region described here an hDII-b splice variant of the same sequence (GenBank accession no. AB041843) was cloned from a human umbilical vein endothelial cell line ECV304 by Ohta et al. (45). They also demonstrated the presence of the hDII-b transcript by RT-PCR in the human brain, lung, kidney, heart, and trachea. Another hD2 mRNA with a 242-bp-long genomic insertion (hDII-c) has also been cloned from ECV304 cells (GenBank accession no. AB041844) (45). We could not detect this transcript in human thyroid by RT-PCR.

Two other D2 mRNA variants have been cloned. In a mouse cochlear cDNA library, a clone was identified containing a truncated D2 coding region followed by the 3’UTR (GenBank AF177197) (42). Interestingly, in this case the divergence point was 35 bp 3’ to the conserved exon/intron junction. The transcript abundance and its functional properties were not reported. The hD2 5’UTR contains an alternatively spliced intron in human thyroid (11). The last four nucleotides of hORF-B and the whole region encoding hORF-C are missing from this D2 mRNA but the potent hORF-A is not affected.

In summary, a number of posttranslational mechanisms can regulate the D2 content of a given tissue. Selenium deficiency reduces D2 activity approximately 30-fold in the mesotheloma cell line MSTD-211 (6). Substrate-induced acceleration of D2 ubiquitination leads to rapid proteasomal degradation, reducing the D2 protein half-life 2- to 3-fold (18). The present studies show that sORFs in the 5’UTR reduce D2 expression by as much as 5-fold. Alternative splicing is another mechanism that dictates the D2 concentration in specific tissues, such as the brain, liver, and thyroid of chickens or humans. While the long, approximately 5-kb 3’UT separating the UGA in the D2 active center from the SECIS element might have been expected to reduce the D2 activity/mRNA ratio by reducing translation, our studies suggest that the AUUUA instability motifs and, more importantly, reducing the distance between the SECIS and coding region may decrease D2 expression by destabilizing the D2 mRNA. However, more direct studies of translational efficiency will be required to confirm this.

The multiplicity of potential regulatory mechanisms for this protein argues that there is meticulous control of the deiodinative activation of T4 to T3. The demonstration that much of this occurs at a posttranscriptional level indicates that D2 activity in different tissues cannot be inferred simply by quantitating its mRNA.

**MATERIALS AND METHODS**

**DNA Transfection**

Cells were transfected as previously described using calcium phosphate precipitation (46). GH4C1 cells were transfected using Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD). Ten micrograms of pUHD10-3 based vector encoding the deiodinase was transiently transfected together with 4 μg pUHD-15, which is required for the transcriptional activation of the pUHD10-3 promoter. This activation can be suppressed by tetracycline that inhibits the binding of the activator expressed by pUHD-15 to the pUHD10-3 promoter (47). The transfection efficiency was monitored by the co-transfection of 3 μg TKGH and hGH was assayed in the media and used to correct D2 or D1 activities (48). Results are given as the mean ± SEM of D2 or D1 activities of duplicate plates of at least three separate experiments as the percentage of the cD2 or rD1 control, respectively.

**Expression Constructs**

The cD2 reporter construct contained a 33-bp cD2 5’UTR fragment as spacer between the cloning site and the initiator ATG followed by the cD2 coding region between EcoRI-HindIII and a rD1 minimal SECIS element between HindIII-NotI (21). In the constructs described below, different UTR fragments were cloned between the SacI site of the D10 vector and RI if not indicated otherwise. The human 5’UTR Vent PCR fragments were generated from the hD2 clone 2 (11). The name of the construct is followed by the oligos in parentheses used to prepare it: hDII-5’UT (Bp35-Bp37); hDII-5’UTΔ (Bp36-Bp37); hORF-A,B-cD2 (Bp36-Bp38); hORF-A-cD2 (SacII-Apol fragment of the Bp36-Bp38 PCR); hORF-B-cD2 (Apol-EcoRI fragment of the Bp36-Bp38 PCR with the Apol at 5’); hORF-C-cD2 (Bp39-Bp37); hDII-5’UTΔ (Bp41-240)-cD2 (Bp36-Bp60); hORF-A(Wt)-cD2 (Bp52 was ligated to the corresponding antisense, latter was not indicated in Table 2); hORF-A(Mut-ATG)-cD2 (Bp54 and corresponding antisense); hORF-A(Mut-Koz)-cD2 (Bp62 and corresponding antisense); cORF-B(Wt)-cD2 (Bp68 and corresponding antisense); cORF-B(Mut-ATG)-cD2 (Bp70 and corresponding antisense).

The hORF-A(Wt)-cD2 and hORF-A(Mut-ATG)-cD2 were cut by EcoRI-NotI and religated, generating in this way hORF-A(Wt)-D10 and hORF-A(Mut-ATG)-D10, respectively. As the rD1 reporter a construct containing a rD1 coding sequence followed by rat D1 minimal SECIS element was used (49). The hORF-A(Wt)-cD2 and hORF-A(Mut-ATG)-cD2 were cut by EcoRI-HindIII and the EcoRI-HindIII fragment of the rD1 reporter (the rD1 coding region) was ligated to generate the hORF-A(Wt)-rD1 and the hORF-A(Mut-ATG)-rD1, respec-

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indicated by arrows were cloned and their sequences are shown in panel A as cD2-clone no. 1 (wt) and cD2-clone no. 2 (Δ77cD2). The positive control (pos. ctr.) clones were plasmids containing the cD2wt and spliced (Δ77cD2) cD2 coding regions. As a negative control, (neg. ctr.) water was used instead of mRNA in the PCR.
Fig. 8. Sequence and Expression of the hD2+108 Transcript

A, PCR of an alternatively spliced hD2+108 transcript from human thyroid. The RNA was isolated from thyroid tissue of a patient with a thyrotropin-producing pituitary adenoma (lane 1) or Graves' disease (lane 2) using the intron spanning Bp97-Bp41 oligos (sequence indicated in Table 2). Both the expected 491-bp amplicon and an approximately 600-bp product were generated. The sequences are shown in panel B. B, Nucleic acid and deduced amino acid sequence of the cloned thyroidal hD2+108 cDNA. The 108-bp intronic sequence inserted in frame between the exon/intron junction of the hD2 coding region are bold and underlined. The inserted sequence contains an in-frame UGA (boxed). A HindIII site was used for assembling the cDNA as described in Materials and Methods. Position 1 is the start of the coding region. C, Immunoprecipitation of Na$_{2}$(75Se)O$_{3}$ labeled HEK-293 cells transfected with hD2+108-SelP SECIS (lane 1) or N-FLAG hD2+108-SelP SECIS (lane 2) using an anti-FLAG antibody. Immunoprecipitate was resolved by SDS-PAGE followed by autoradiography for 12 d. N-FLAG hD2+108-SelP SECIS expresses a D2 protein of approximately 35 kDa showing that the inserted UGA is translated. No 35-kDa band was present in lane 1, indicating that the FLAG IP is specific. D, Genomic organization of the alternatively spliced hdlo2 gene. The 108-bp fragment from the intron is inserted between codons 74 and 75 (see panel B).
The hD2+108 Coding Region

The mRNA from thyroid of two patients, one with a thyrotopin-producing pituitary adenoma or Graves' disease and a normal thyroid obtained postmortem were isolated by Trizol (Life Technologies, Inc.). Reverse transcription was started by a 3-UTR sample, ligated into pGEM-T (Promega Corp.) (5' at T7). A Sacl-HindIII fragment (the latter from the insert) was placed between these sites of the Bp97-Bp41 insert producing the hD2+108 coding in pGEM-T (hD2+106pGEM-T). To place the insert 5' to a SECIS element in the D10 vector the Sphi-PstI fragment of the hD2+108pGEM-T was inserted between these sites of hD2-D10-SelP, respectively. The constructs were sequenced in the ABI Prism 377 automated sequencer using dye terminators.

Deiodinase Assays

Homogenates of transfected cells were assayed in duplicate for either D2 or D1 activity as described (21). In brief, D2 assays contained $[125]^{1}T_{4}$, 2 nm T4, and 20 mM dithiothreitol (DTT) in phosphate-EDTA (PE) buffer while for D1 measurements $[125]^{1}T_{3}$, 1 μM T3, and 10 mM DTT was used in a 300-μl volume. Activity was expressed as pmol/min-mg protein. A telencephalon sample from an adult chicken was homogenized in PE buffer containing 0.25 sucrose and 10 mM DTT. The sample was taken from the same animal used earlier for liver D2 measurements (21). For the maximum velocity and Michaelis-Menten constant ($T_{m}$) determination, 200 μg protein were assayed in duplicate for 5'-deiodinase activity in a final volume of 300 μl PE buffer containing 20 mM DTT, $[125]^{1}T_{3}$, 1, 1.5, 3, and 10 mM T4 and 1 mM 6-propylthiouracil.

### Table 2. PCR Primers Used for Cloning and Generation of Constructs and Probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense/Antisense</th>
<th>Orientation</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bp3</td>
<td>Sense</td>
<td>CTGAATTCAT CCGGCGAGAG AGAG</td>
<td></td>
</tr>
<tr>
<td>Bp11</td>
<td>Antisense</td>
<td>CTCACCCAGAA GCCGCAGAAG G</td>
<td></td>
</tr>
<tr>
<td>Bp35</td>
<td>Sense</td>
<td>cattctctcg ggcgccggtT CCTGCAAAGA GAGGGCATT G</td>
<td></td>
</tr>
<tr>
<td>Bp36</td>
<td>Sense</td>
<td>cattctctcg ggcgccggtG GAAGGGAAA ACAGAGAGTG AG</td>
<td></td>
</tr>
<tr>
<td>Bp37</td>
<td>Antisense</td>
<td>ggatctccT CTCTGCTCCT CGTAGTCAG G</td>
<td></td>
</tr>
<tr>
<td>Bp38</td>
<td>Antisense</td>
<td>ggatctccAG CTGGCGTACT CGTCCCTAAT G</td>
<td></td>
</tr>
<tr>
<td>Bp39</td>
<td>Sense</td>
<td>cattctctcg ggcgccggtT GAATGATTTG ATGGGACACG TC</td>
<td></td>
</tr>
<tr>
<td>Bp41</td>
<td>Antisense</td>
<td>GCCTCATCAG TTGAGACAG CA</td>
<td></td>
</tr>
<tr>
<td>Bp52</td>
<td>Sense</td>
<td>tccgccggtG ATAGAGCAGA TGATAGCTA AAgaatcc</td>
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</tr>
<tr>
<td>Bp54</td>
<td>Sense</td>
<td>tccgccggtG ATAGAGCAGA TGATAGCTA AAgaatcc</td>
<td></td>
</tr>
<tr>
<td>Bp60</td>
<td>Antisense</td>
<td>ggatctccT TTTAAGCTGA GCCGGTCTT</td>
<td></td>
</tr>
<tr>
<td>Bp62</td>
<td>Sense</td>
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<tr>
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<td>Sense</td>
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<tr>
<td>Bp90</td>
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<tr>
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<td>Sense</td>
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<tr>
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<td>Sense</td>
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<td>Bp108</td>
<td>Antisense</td>
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<tr>
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<td>TTAACCGGT AATCTAGTGT TCTTTCTC</td>
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<tr>
<td>Bp112</td>
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<tr>
<td>Bp113</td>
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<tr>
<td>Bp115</td>
<td>Sense</td>
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<tr>
<td>Bp135</td>
<td>Sense</td>
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<tr>
<td>Bp136</td>
<td>Antisense</td>
<td>GCATATTCTG TGGAGGGCTA TACTGT</td>
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</tbody>
</table>

Non-gene-specific tails or mutations are indicated in lowercase letters.
Incubation was for 120 min at 37 °C. Activity was expressed as fmol T$_4$ deiodinated/hr/mg protein.

**Northern Blots**

D10 based constructs were transfected into HEK-293 cells as described. On the second day after transfection, 1 μg/ml tetracycline was added to the culture medium to block transcription (47). Cells were harvested after 13 h after tetracycline addition and RNA was isolated using Trizol LS (Life Technologies, Inc.). RNA was treated using ribonuclease-free deoxyribonuclease I (Life Technologies, Inc.), following the instructions of the manufacturer. Ten micrograms of total RNA were processed for Northern blot. The experiment was performed on two separate samples for each construct. The blot was probed with a 450-bp cDNA region (21) then stripped and probed with a 330-bp probe generated by PCR (bp135-bp196) containing the SE10S region of cD2. The blot was exposed for 20 h at −80 °C on Biomax film (Eastman Kodak, Rochester, NY). Northern blots were performed as described (21). As denominator for densitometry, both the density of the 28S subunit and the amount of the hGH in the media were used to monitor loading and transfection efficiency, respectively.

**Na$_2$[35Se]O$_4$ Labeling and Immunoprecipitation of Transiently Expressed FLAG-D2**

The FLAG D2+/108-SEI P or D2+/108-SEI P constructs were transfected into HEK-293 cells. The transfected cells were cultured in the presence of 4–6 μCi of Na$_2$[35Se]O$_4$/dish on d 2 after transfection in the presence of DMEM supplemented with 10% fetal bovine serum. Na$_2$[35Se]O$_4$ was kindly provided by the University of Missouri Research Reactor, courtesy of Drs. Marla Berry and Dolph L. Hatfield. On d 3, the cells were washed with PBS and sonicated in a lysis buffer [1% Triton X-100, 1% bovine hemoglobin, 0.2 U aprotinin/ml, 0.2 U leupeptin/ml 1 mM phenylmethylsulfonyl fluoride in Tris-saline-azide (TSA) buffer (0.01 M Tris-HCl, pH 8.0; 0.14 M NaCl)] Sonication was followed by lysis under slow agitation at 4 C for 30 min. After centrifugation of the lysate at 4000 rpm for 15 min, each supernatant was incubated under slow agitation at 4 C for 1 h with preimmune mouse sera to a final dilution of 1:100 and 20 μg protein.

**Sequences**

The chicken and human 5′UTR are formed in the GenBank accession nos. AF125575 and AF188709 (11, 21). The missing 5′ portion of the mouse 5′UTR was recently published by Song et al. (44). The rat D2 5′UTR is contained in the GenBank entry nos. U53505 and AF249274 (1, 12). The TSS of the chicken and mouse D2 mRNA was determined by 5′RACE and of the human by 5′RACE, S1 digestion and primer extension (11, 21, 44). The putative TSS of rat D2 was assigned by sequence alignment. The sequence of the Δ77cD2 coding region was deposited into the GenBank under accession no. AF401753.

**Acknowledgments**

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