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Regulatory Role of the 3' Untranslated Region (3'UTR) of Rat 5' Deiodinase (D1). Effects on Messenger RNA Translation and Stability

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The previous findings that both a long and a short type 1 deiodinase (D1) mRNA are present in different tissues and that the D1 gene contains two potential polyA signals suggest that the two mRNAs result from differential polyA signal usage. In this study, we examined the properties of the two D1 mRNAs generated in HEK 293 cells by the alternative use of each of the poly A signals in order to ascertain the potential regulatory role of the 3'UTR of this gene. Our results showed that the long mRNA is less stable, but that it is translated more efficiently than the short mRNA. The net result of these differences is a higher D1 activity with the long message. These data suggest that the D1 3'UTR may play an important role in regulating the stability and translational efficiency of the D1 mRNA, both of which could be physiologically relevant when the demand for D1 activity is high.

Key Words: Deiodinases; 3' untranslated region (3'UTR); polyadenylation signals; translation efficiency; RNA stability.

Introduction

The peripheral conversion of the prohormone thyroxine (T4) to the active hormone 3,5,3'-triiodothyronine (T3) is catalyzed by two different selenoenzymes denominated type 1 (D1) and type 2 deiodinase (D2). D1 provides most of the circulating T3 and is expressed predominantly in liver, kidney, and thyroid gland, although it has also been detected in pituitary, heart, and mammary gland *(1–3)*. Neither of these enzymes has yet been purified. An incomplete D1 mRNA was first described in 1990 *(4)*. This transcript, obtained from euthyroid rat liver, contained only part of the 5' untranslated region (5'UTR) and had two potential stop codons and one polyadenylation (polyA) signal site (ATTAAA). One year later, Berry et al. *(5)* described in hyperthyroid rat liver a complete D1 mRNA of 2094 basepairs (bp), with one stop signal (TGA) that also encoded for the amino acid selenocysteine and a second true stop codon (TAG). This transcript was designated G21 and has two potential polyA signal sites, the first at 1612 bp (ATTAAA) and the second at 2069 bp (TATAAA). Recently, we reported that liver, heart, and lactating mammary gland express two different D1 mRNA species, one of 2.1 kb, which is identical in size to G21, and a shorter message of 1.6 kb similar to that described by St. Germain and co-workers in 1990 *(4,6)*. These messages probably result from differential usage of the second and the first polyA signals, respectively *(2)*. Moreover, we showed that the expression of the long mRNA was detected only under catabolic conditions such as hyperthyroidism, β-adrenergic stimulation, or vigorous suckling stimulation, whereas the shorter mRNA was continuously present in the same tissues *(6)*.

Studies of other transcripts have demonstrated that the 3'UTR is involved in the control of translation efficiency as well as in mRNA stability, and that it constitutes a site of functional regulation *(7–9)*. In relation to the translation efficiency, it is known that approx 80% of mature mRNAs use the conserved AATAAA signal, which is called the canonical polyA site *(10)*. Other sequences, however, have also been identified as polyA signals that may have a positive or negative influence on the mRNA translation efficiency *(11)*. The 3'UTR may contain other important signals, and one such signal that is deleterious for mRNA stability is the presence of AUUUA motifs in the 3'UTR. These sequences, called AU-rich regions (AREs), may mediate the translation-dependent assembly of a large mRNA-destabilizing complex that induces rapid degradation of mRNAs *(12)*.

The purpose of this study was to determine the functional significance of the two polyA signals contained in the 3' UTR of D1 mRNA. Two D1 mRNAs were generated in human embryonic kidney cells (HEK293) using either the first (ATTAAA) or the second (TATAAA) polyA signal.

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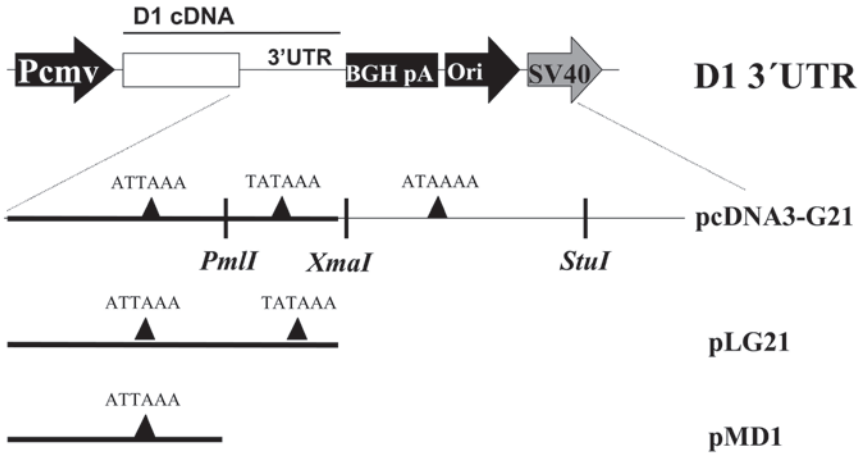


Fig. 1. Schematic representation of plasmid containing the pcDNA3-G21 and its derivatives. ATTAAA and TATAAA, sequences and positions of D1 polyA signals; AATAAA, canonical polyA site of BGHpA; pcDNA3-G21, plasmid with the full length D1 cDNA (2.1 kb), and internal BGH polyA signals; pLG21, with two D1 polyA signals; pMD1, with only the upstream D1 polyA signal.

While both messages were present at apparently similar steady-state levels, five times more D1 activity was detected when the longer transcript was generated using the second polyA site. The half-life of the longer mRNA, however, was significantly shorter than that of the short mRNA, suggesting that the increase in enzymatic activity is not related to mRNA stability but to increased efficiency of translation. The instability of the long mRNA is consistent with the presence of two AREs near the second polyA signal. Together, these data indicate that the D1 3'UTR may play a significant role in regulating the stability and the translational efficiency of D1 mRNA.

Results

Both D1 PolyA Signals Are Functional and Generate Long and Short mRNAs

To determine whether the D1 polyA signals are functional, constructs containing the D1 coding sequence with only the first (ATTAAA) or with both (ATTAAA and TAT AAA) polyA signals (designated pMD1 and pLG21, respectively, Fig. 1) were transfected into HEK293 cells, and their products were analyzed. The mRNA derived from pMD1 was expected to yield a message corresponding to the endogenous short D1 mRNA using the first polyA signal, and the mRNA derived from pLG21 was expected to yield a long transcript using the second polyA signal as described previously *(5)*. To test these predictions, a Northern blot analysis was performed with a probe from the D1 coding region that would detect both possible D1 mRNA species (Fig. 2). As expected, pMD1 yielded a short D1 mRNA, while pLG21 yielded along mRNA. The presence of only one band in each sample indicates that both polyA signals are functional and, in the case of the long mRNA, that the second signal is used. The 3' end of the long message generated from the vector has been shown to correspond exactly to the sequence

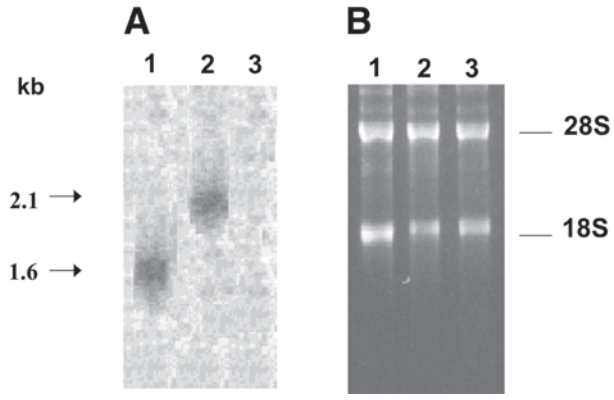
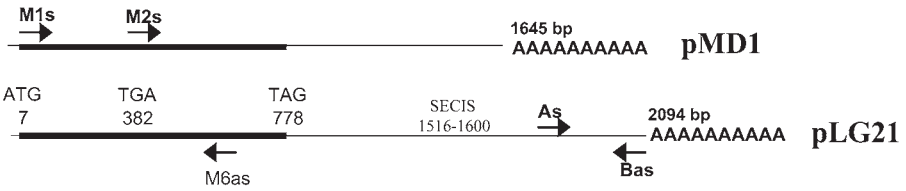


Fig. 2. Northern blot analysis of D1 mRNAs. (**A**) Total RNA of HEK293 cells transfected with the vector pMD1 (lane 1), pLG21 (lane 2), and mock-transfected (lane 3) probed with a radiolabeled D1 cDNA fragment (M1s-M6as amplicon) common to both D1 mRNAs. (**B**) Denaturing RNA gel stained with ethidium bromide, showing the integrity of these RNA samples.

of the long message found in vivo in hyperthyroid liver tissue *(5)*. In the case of the short message generated in vitro in this study, we sequenced its 3' end and found that it contained eight nucleotides more than the corresponding message expressed in liver, mammary gland, and heart, which we sequenced in a previous study *(6)*. The *Pml*1 enzyme cut the G21 plasmid eight nucleotides downstream from where the endogenous short messenger ended. Hence, the messages generated in vitro closely match those found in vivo.

Additional experiments were performed using a previously developed semiquantitative RT-PCR method *(2)* to measure the expression of both D1 mRNA species. The relative location and size of the oligonucleotides used are summarized in Fig. 3. Amplification of GAPDH was performed in parallel to monitor RNA extraction and cDNA amplification efficiency. This method revealed no differ-

A



B

Primer	Size	Location	Sequence	Purpose
M1s	17-mer	66-82	CTT GGA GGT GGC TAC GG	PCR
M2s	20-mer	377-396	GCA CCT GAC CTT CAT TTC TT	PCR
M6as	18-mer	610-627	CTG GCT GCT CTG GTT CTG	PCR
As	20-mer	1693-1712	CTG CCG TCT TTT CCG ATA CG	PCR
Bas	20-mer	1809-1829	CCC TTC CAC AAC ACT TCA CA	PCR

C

Primer	Size	Location	Sequence	Purpose
c-mycs	20 mer	1539-1614	AGA GAA GCT GGC CTC CTA CC	PCR
c-mycas	20 mer	3749-3768	AGC TTT TGC TCC TCT GCT TG	PCR
GAs	26 mer	497-472	TGA AGG TCG TGT TCA ACA GAT TTG GC	PCR
GAas	24 mer	994-1017	CAT GTA GGC CAT GAG GTC CAC CAC	PCR

Fig. 3. (**A**) Schematic representation of the two D1 mRNAs. SECIS: selenocysteine insertion sequence element. (**B**) Oligonucleotides used for amplification of D1. (**C**) Oligonucleotides used for GAPDH (GA) and c-myc mRNAs.

ences in D1 mRNA abundance in HEK293 cells transfected with pMD1 or pLG21 (Figs. 4A,B).

The Long D1 mRNA Is Translated at a Higher Rate than the Short D1 mRNA

To assess possible differences in the rate of translation of the pMD1 and pLG21 mRNAs, these two constructs were cotransfected into HEK293 cells with a control plasmid encoding the β-galactosidase reporter gene. After 24 h, the cells were collected, and D1 and β-galactosidase enzymatic activities were assayed. The level of D1 activity was normalized to that of the β-galactosidase activity to control for transfection efficiency and protein concentration. As shown in Fig. 5A, both constructs yielded D1 activity, which was undetectable in mock-transfected HEK293 cells. Transfection with pLG21, which uses the second polyA signal, yielded five times more D1 activity than the transfection with pMD1, which uses the first polyA signal. Analysis by RT-PCR indicated that, in keeping with the results described above and with those obtained when each D1 vector was transfected alone, the abundance of the two D1 mRNAs was similar (Fig. 5B). This suggests that differences in the enzymatic D1 activity generated by the two vectors is the result of their differential rate of translation. That is, the long mRNA (pLG21) appears to be translated more efficiently than the short mRNA (pMD1).

Long and Short D1 mRNAs Have Different Stability

To assess possible effects of the D1 3'UTR on mRNA stability, we determined, by RT-PCR and Northern blot, the half-life of the pLG21 and pMD1 mRNAs in HEK293 cells after RNA synthesis was blocked by treatment with act-D (Fig. 6). The half-life of the short mRNA (pMD1) was 14.4 h, while that of the long mRNA (pLG21) was 5.9 h (Figs. 6A,B). These half-life estimates were corroborated by Northern blot analysis (Fig. 6C).

To ensure that transcriptional shut-off was efficient, RT-PCR was carried out to detect c-myc and GAPDH mRNAs as controls for short (15–40 min) and long (more than 24 h) half-lives, respectively. As expected, no c-myc mRNA was detected 4 h after act-D treatment, while GAPDH mRNA was detected even after 16 h of the drug treatment (Fig. 6A).

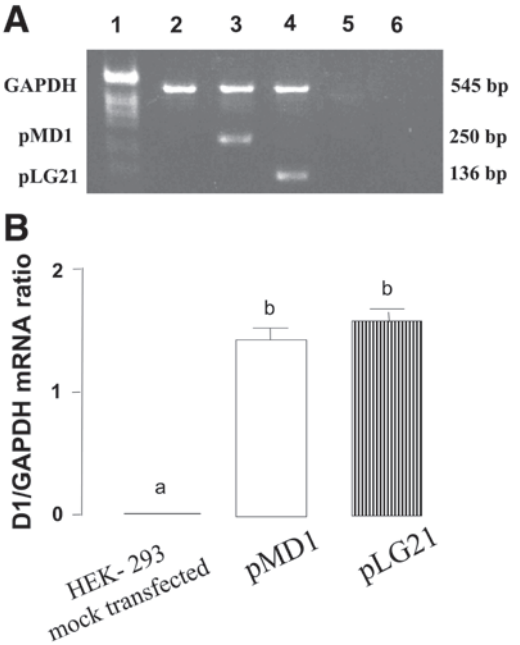


Fig. 4. Determination of steady-state levels of D1 mRNA in HEK293 cells. (**A**) Representative electrophoresis of the semi-quantitative RT-PCR products based on the primers used. The PCR fragments expected were 545 bp for the endogenous enzyme GAPDH, 250 bp for the short D1 mRNA, and 136 bp for the long D1 mRNA from cells transfected with pMD1 or pLG21, respectively. Lane 1, molecular weight ladder; lane 2, mock-transfected cells; lane 3, cells transfected with pMD1; lane 4, cells transfected with pLG21; lane 5, RNA sample and the appropriate oligonucleotide primers but without reverse transcriptase; lane 6, water with all the PCR reagents. (**B**) Densitometric values normalized with GAPDH expression (D1/GAPDH). Values represent mean ± SD from three independent transfections. Means with different letters indicate significant differences ($p < 0.05$).

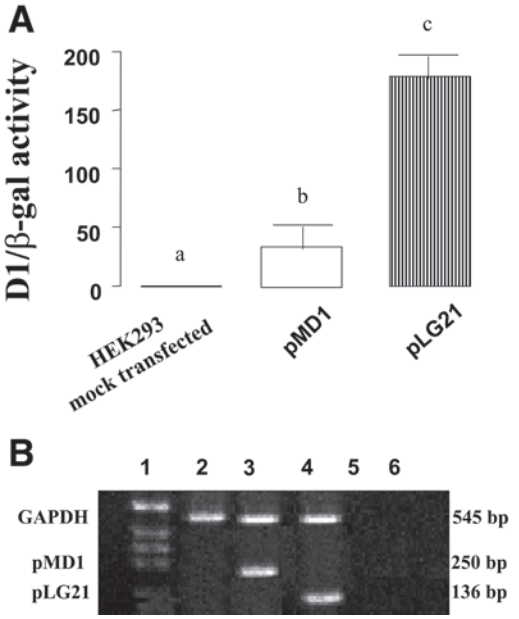


Fig. 5. 5' deiodinase activity in HEK293 cells. (**A**) 5' deiodinase and β-galactosidase activity were assayed using protein extract prepared from cells cotransfected with a plasmid encoding the β-galactosidase gene (pRSV-β-gal) and pMD1 or pLG21. The value obtained for 5' deiodinase activity was normalized to the β-galactosidase activity and plotted as D1/ β-gal activity. Values indicate the mean ± SD from five independent transfections. Data were analyzed with a one-way ANOVA and Kruskal–Wallis test. Means with different letters indicate significant differences ($p < 0.05$). (**B**) Electrophoresis of PCR products. The PCR fragments expected were 545 bp for GAPDH, 250 bp for the short D1 mRNA, and 136 bp for the long D1 mRNA from cells co-transfected with pMD1 or pLG21 and β-gal. Lane 1, molecular weight ladder; lane 2, mock-transfected cells; lane 3, transfected cells with pMD1+ β-gal; lane 4, transfected cells with pLG21+ β-gal; lane 5, RNA sample and the appropriate oligonucleotide primers but without reverse transcriptase; lane 6, water with all the PCR reagents.

Discussion

Two different D1 transcripts have been previously detected in liver, heart, and mammary gland *(2,4–6)*. We have proposed that these variants, which have sizes of 2.1 and 1.6 kb, result from differential use of two alternative polyA signals located at the 3' end of the gene *(2)*. The use of the first polyA signal (ATTAAA) would generate the short D1 mRNA (1.6 kb), and the second polyA signal (TATAAA) would generate the long D1 mRNA (2.1 kb). In this work we studied the effects of the 3'UTR sequence on the properties of the D1 mRNA species in cultured HEK293 cells. Our results show that both polyA signals can generate mature mRNAs and protein (D1 enzyme), which means that both are functional. The use of the first signal can be forced by deletion of the second one and, in agreement with other reports, the second signal is used preferentially when both signals are present *(13,14)*.

The long and short mRNAs were expressed at similar steady-state levels from the vectors used, and, yet, the long message yielded a higher D1 activity than the short message. This suggests that the long message is translated more efficiently. The lower efficiency of translation associated with the first signal (ATTAAA) is consistent with studies in which a point mutation in the canonical signal to convert it to ATT AAA was accompanied by a drastic decrease in translation efficiency *(11)*. Although the precise regulatory mechanisms involving polyA signals are not well understood, it has been demonstrated that these sequences participate in the recruitment of proteins involved in the circularization of mRNA molecules necessary for initiation of translation and modulation of the rate of translation *(15–20)*.

Our findings are consistent with previous observations that link the presence of the long mRNA to catabolic situations of increased demand for T_3, the main product of D1, while the short message is continuously present in heart, liver, and mammary gland *(2)*. The long D1 mRNA was found to be augmented by hyperthyroidism, supraphysiological administration of an alpha adrenergic agonist, or vigorous resuckling after 12 h of nonsuckling in mammary gland *(2,6)*. Moreover, it has been shown in immortalized FRTL-5 rat thyroid cells that a 2.1 kb D1 mRNA is synthe-

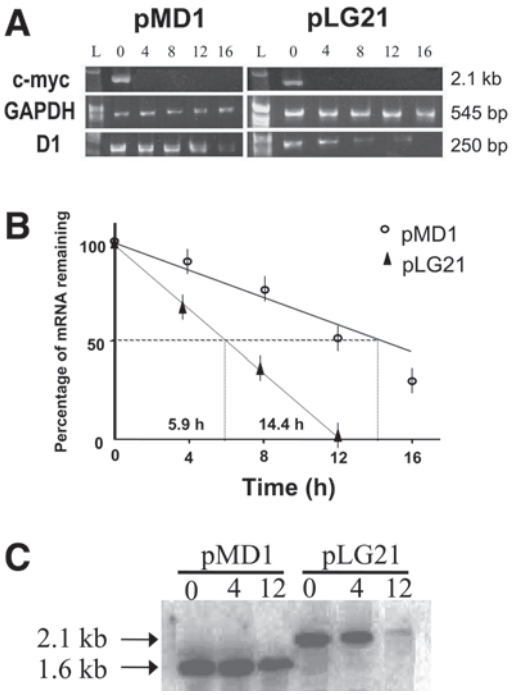


Fig. 6. Stability of short and long D1 transcripts. (**A**) Electrophoresis of PCR products. At 24 h post-transfection of D1 vectors into HEK293 cells, act-D was added to determine the half-life of the D1, c-myc, and GAPDH mRNAs. L, molecular weight ladder. (**B**) Graphical representation of the mRNA decay and calculation of half-lives. The amount of mRNA observed at the first time point of act-D treatment was normalized to 100%, and the percentage of remaining signal is plotted vs time (h). The calculated half-life of pMD1 mRNA is 14.4 h, whereas that of pLG21 is 5.9 h. Each point represents an average value calculated from three independent experiments. (**C**) Northern blot analysis of 10 µg total RNA from pMD1 and pLG21 was carried out with samples from 0, 4, and 12 h after act-D. The calculated half-life values (n = 2) were 14.2 h for pMD1 and 6.1 h for pLG21.

sized only after thyroid stimulating hormone (TSH) or T_3 treatment *(21)*. When D1 mRNA sequences from other species were compared, we found that the fish tilapia mRNA also contains two polyA signals *(22)*, suggesting that the presence of two mRNAs for the D1 enzyme could be widespread throughout vertebrates. The presence of D1 mRNA with only one signal in other species *(5,6,22–26)* might reflect the metabolic conditions of the animal from which the samples were obtained.

In additional experiments, we studied the effect of the D1 3'UTR on mRNA stability. Using 10 µg/mL of act-D to shut down transcription, we found that the short D1 mRNA is more stable than the long mRNA, with half-lives of 14.4 and 5.9 h, respectively. As described above, a 2.1 kb D1 mRNA is induced in FRTL-5 cells by TSH and T_3 *(21)*. Analysis of its rate of disappearance after transcription shut down with 5 µg/mL act-D revealed a half-life of approx 16 h. The apparent discrepancy between that determination and our results could reflect differences between the different cell lines used or may be due in part to the different concentrations of act-D used in the two studies. When we used 5 µg/mL act-D to block transcription as in the study by Toyoda et al. *(21)*, we found half-lives of 17.7 and 24.5 h for the long and the short messages, respectively (data not shown). Moreover, under similar experimental conditions, the c-myc mRNA could be readily detected at least up to 8 h after addition of the drug, which conflicts with its known half-life of 40 min *(27)*. These results suggest that the act-D concentration used in the earlier study *(21)* was not sufficient to shut down transcription completely, hence resulting in an apparently greater half-life of the D1 long mRNA.

Regulatory elements playing a role in the stability and the turnover of polyA RNA have been identified in the 3'UTR of several genes *(28)*. The presence of AREs, which are RNA-destabilizing elements in a wide variety of short-lived mRNAs including those encoding proto-oncoproteins, nuclear transcription factors, and cytokines, suggest a critical role of AREs in regulating gene expression *(29–32)*. D1 mRNA contains two AREs (AUUUA motifs) about 100 nucleotides upstream from the second polyA signal and, hence, they are only present in the less stable, long D1 mRNA. Because there are no such motifs in the region upstream to the first polyA site, we postulate that the lower stability of the long mRNA is causally linked to these AREs. Further analysis, however, will be necessary to confirm this notion.

Differential usage of two polyA signals has also been detected in other mRNAs such as those encoding MnSOD *(33)*, the amyloid precursor protein *(34)*, the human PR264/SC35 splicing factor *(8)*, the *Xenopus* α-tubulin mRNA *(7)*, and the mouse *kappa* opioid receptor *(35)*. The physiological relevance of the alternate forms of these mRNAs and how their generation is regulated, however, remain largely unknown. We showed that the two forms of the D1 message are expressed at similar levels but have different stabilities. Moreover, the enzymatic activity was higher with the less stable mRNA, suggesting that the two mRNAs also have different translation efficiencies. We propose that these differences are linked with diverse metabolic conditions and demands for T_3, the primary product of D1. The higher translation efficiency of the long message might be relevant under high metabolic demand when it is differentially synthesized, and its lower stability might be important for a rapid return of T_3 synthesis to a basal level under normal metabolic conditions *(6)*. Additional studies combining in vitro and in vivo methods will be required to ascertain the mechanisms involved in the regulation of the synthesis of each D1 mRNA and to test the physiological relevance of this regulation.

Materials and Methods

Plasmid Construction

D1 expression vectors were constructed using the Blue Script-G21 cDNA clone *(5)*, which contains the 2.1 Kb full

length D1 coding region and its 3'UTR. The cDNA was subcloned into the *Kpn*I/*Xba*I sites of pcDNA3 to generate pcDNA3-G21 (Fig. 1). This vector contained, in addition to the two D1 polyA signals, the BGH polyA signal from the original pcDNA3. The BGH signal was eliminated by excision of a 1206 nt fragment by digestion with *Pml*I/*Xma*I. The digested plasmid was religated with T_4-DNA ligase (Gibco-BRL, Gaithersburg, MD) to yield the vector pLG21 containing only the two D1 polyA signals. The pMD1 vector containing only the upstream D1 polyA signal was constructed by excising from pcDNA3-G21 a 1572 nt *Pml*I/*Stu*I fragment that contained the downstream D1 and BGH polyA signals; the protruding ends were made blunt by treatment with the large fragment of DNA polymerase I (Invitrogen, Carlsbad, CA). The blunt-end vector was religated using T_4-DNA ligase. The plasmid constructs were verified by sequencing. Long mRNA derived form this vector is identical to the endogenous liver mRNA described by Berry et al. *(5)*, and the short mRNA derived from our vector is 8 nucleotides longer in the 3'UTR than that from lactating mammary gland *(2)*.

Cell Culture and Transfection

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% CO2. For transfections, cells were seeded in six-well plates (3×10^5 cells/well) (Corning, NY). When the cells reached 75–80% confluence, transfection was performed with the FuGENE6 reagent (ROCHE, Indianapolis, IN) using 1 µg of either pMD1 or pLG21 plasmid DNA and 200 ng of pRSV-βgal, which carries the β-galactosidase (β-gal) gene as a reporter for monitoring transfection efficiency.

Measurement of 5' Deiodinase and β-Galactosidase Activity

5' deiodinase activity was determined by a modification of the release of radiolabeled iodide method *(36)* optimized for mammary gland *(37)*. The HEK293 cells were homogenized 24 h after transfection with ice-cold lysis buffer (10 m*M* HEPES, pH 7.0, 0.32 *M* sucrose, 1.0 m*M* EDTA, and 10 m*M* DTT), centrifuged at 1000*g* at 4°C to eliminate nonhomogenized cell debris, and frozen at −70°C. The D1 activity was measured in a reaction mixture containing cell homogenate (100 µg protein), 2 n*M* [^{125}I]rT_3, 0.5 µ*M* non-radiolabeled rT_3, and 5 m*M* DTT. Incubations were performed at 37°C for 3 h. The released acid-soluble radioiodide was isolated by chromatography on Dowex 50W-X2 columns. Protein concentration was measured by the Bradford method (Bio-Rad protein assay, Bio-Rad, Richmond, CA). The β-galactosidase Reporter Gene Activity Detection Kit was used to measure β-gal activity as described by the manufacturer (Sigma). All the assays were done in duplicate, and all D1 determinations were normalized to β-gal activity.

Semiquantitative RT-PCR

Total RNA was extracted from cell lysates using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Quantitation of D1 mRNA was performed as described *(6)*. One microgram of total RNA from transfected or mock-transfected HEK293 cells was treated with DNAse I before it was reverse transcribed using Superscript™ II reverse transcriptase (RT) (Invitrogen) and oligo (dT) as primer. One microliter of the total RT product was used in each of the subsequent PCR reactions with the following primer combinations: for both short and long D1 mRNAs, M2s and M6as (Figs. 3A,B); for the long D1 mRNA, As and Bas; and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GAs and GAas (Fig. 3C). PCR reactions were carried out for 25 cycles, because, as previously demonstrated *(6)*, amplification is still in the linear phase, which is essential for the semiquantitation. Each cycle consisted of melting at 94°C for 45 s, annealing at 54.5°C for 45 s, and extension at 72°C for 1 min. As a control, a reaction mixture containing an RNA sample with the appropriate oligonucleotide primers, but without the reverse transcriptase (RT–) was included in every experiment. The PCR fragments were 250 bp for the short D1 mRNA, 136 bp for the long D1 mRNA, and 545 bp for GAPDH, and they were resolved in a 2% agarose gel and visualized using ethidium bromide. The sizes of the bands were confirmed by using a commercial DNA ladder (1 kb DNA ladder, GIBCO-BRL). Polaroid pictures were taken and digitized using a Hewlett Packard Scanner Jet 11CX, and the signals were analyzed by using an editing version of the NIH-image program. The values obtained were normalized according to the GAPDH mRNA levels detected in each sample.

Northern Blots

Ten microgram samples of total RNA were electrophoresed in 1% agarose formaldehyde denaturing gels and transferred to nylon membranes. The transferred RNA was fixed to the membrane using a UV cross-linker (Stratalinker; Stratagene, La Jolla, CA) at 12 mJ and prehybridized for 3 h in a solution containing 0.75 *M* NaCl, 0.05 *M* TES, 0.05 *M* EDTA (pH 7.1), 1X Denhardt's solution, 50% deionized formamide, and 0.5 mg/mL salmon sperm DNA at 37°C. The D1 cDNA probes were radiolabeled using [α-^{32}P]dCTP (Amershan Biosciences) and the Prime-It®II Random Primer Labeling Kit (Stratagene, La Jolla, CA), and hybridization was performed overnight at 30°C in fresh buffer. The membranes were exposed for 10 h with a phosphor-intensifying screen and scanned in a Storm phosphoimager (Applied Biosystems).

RNA Stability

pMD1 and pLG21 were independently cotransfected into HEK293 cells with pRSV-*Bgal*. Twenty-four hours

after transfection, actinomycin D (act-D) was added to the culture at a final concentration of 10 μg/mL, and total RNA was extracted in independent cultures 0, 4, 8, 12, and 16 h later. Treatment with 10 μg/mL act-D blocked transcription without affecting cell viability (data not shown). RT-PCR amplification was carried out as described previously for the D1 and GAPDH mRNAs. c-myc mRNA was amplified by PCR using 1 μL of RT mixture and the following primers: c-mycs and c-mycas (Fig. 3C). Amplification was carried out for 25 cycles at 92°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The size of the c-myc PCR fragment was 2.1 kb. The PCR products from the D1, GAPDH, and c-myc genes were resolved on a 1.5% agarose gel and visualized with ethidium bromide. The quantities of each product were normalized according to the GAPDH mRNA levels detected in each sample. To confirm the amount of D1 transcripts, one Northern blot analysis from pMD1 and pLG21 total mRNA was carried out with samples from 0, 4, and 12 h after act-D addition.

The half-life of each mRNA was calculated as described *(29)* using the equation $t_{1/2} = \ln 2/k$, where k is the slope derived from the linear equation $\ln C = \ln C_0 - kt$, and C is the concentration of mRNA at time t.

Statistical Analysis

Data are expressed as the mean ± SD. Differences between experimental groups were analyzed using a one-way analysis of variance (ANOVA), Kruskal–Wallis, or 12 Student's t test. Differences with a $p < 0.05$ were considered statistically significant.

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