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Isolation and Characterization of the Mouse Gene for the Type 3 Iodothyronine Deiodinase*

ARTURO HERNÁNDEZ†, GHOLSON J. LYON‡, MARK J. SCHNEIDER, AND DONALD L. ST. GERMAIN

Departments of Medicine and Physiology, Dartmouth Medical School, Lebanon, New Hampshire 03756

ABSTRACT

The type 3 iodothyronine deiodinase (D3) is a selenoenzyme that inactivates thyroid hormones by removing a iodine from the 5-position of the tyrosyl ring. D3 is highly expressed in many tissues during the early stages of development, and its activity is regulated by selected growth factors and various hormones. To gain further insights into the structure, functional role, and regulation of this enzyme, we screened a mouse liver genomic library with a rat D3 complementary DNA probe and isolated a 12-kb clone coding for the *Dio3*. Restriction analysis followed by Southern blotting and nucleotide sequencing demonstrated that the *Dio3* contains a single exon, 1853 bp in length, that encodes the entire length of the messenger RNA expressed in murine placenta and neonatal skin. Primer extension experiments identified two potential transcriptional start sites located 77 and 60 nt upstream of the ATG translational start codon. The region immediately 5′ to the start sites contains consensus TATA, CAAT, and GC elements. Furthermore, a 526-nucleotide genomic fragment from this region was demonstrated to efficiently drive a luciferase reporter construct when transfected into COS-7, XTC-2, or XL-2 cells or into primary cultures of rat preadipocytes derived from neonatal brown fat. In conclusion, D3 transcripts in the placenta and skin are encoded by the *Dio3* gene from a single exon whose expression is regulated by an upstream region that contains several consensus promoter elements. Further characterization of this gene will provide new insights into the factors regulating the unique pattern of D3 expression during development. (*Endocrinology* **140:** 124–130, 1999)

THE TYPES 1, 2, and 3 iodothyronine deiodinases (D1, D2, and D3, respectively) constitute a group of enzymes that metabolize thyroid hormones and thus regulate their levels in many tissues (1). Complementary DNAs from several species that code for the three deiodinase isoforms have been isolated (2–9), and all contain a TGA codon within the open reading frame that is translated as selenocysteine. The incorporation of this uncommon amino acid into the protein is directed by a specific, conserved sequence in the 3′-untranslated (3′-UT) region of the messenger RNA (mRNA) that is termed a selenocysteine insertion sequence (SECIS) (10). Such *cis*-acting elements are a common feature of all selenoprotein mRNAs described to date. Site-directed mutagenesis has demonstrated that the selenocysteine residue is located at the active site of these enzymes and is essential for efficient catalysis (3, 5, 8). Recently, genomic mouse and human DNA fragments containing the D1 gene have been isolated and characterized, and the human gene has been localized to chromosome 1p32-p33 (11, 12).

In contrast to D1 and D2, which catalyze primarily the conversion of T_4 to the more active hormone T_3 by 5′-deiodination, the D3 inactivates T_4 and T_3 by removing an iodine from the 5-position or from the chemically equivalent 3-position on the inner ring (13). D3 is highly expressed during development in many tissues, including placenta, liver, intestine, skin, and central nervous system, whereas in adult mammals expression appears to be restricted primarily to the skin and brain (1). Mammalian D3 complementary DNAs (cDNAs) of approximately 2.1 kb have been isolated from rat neonatal skin (6) and human placenta libraries (7). This size corresponds closely to that of the mRNA species in these tissues that hybridize to the D3 cDNAs. In rat brain, however, larger D3 transcripts of 3.6 and 3.3 kb have been observed (6). cDNAs corresponding to these mRNAs have not yet been identified.

Multiple hormonal and other regulatory factors have been demonstrated to influence D3 expression. Thus, selected growth factors (such as epidermal growth factor and acidic and basic fibroblast growth factors) and phorbol esters markedly induce D3 mRNA expression and activity in certain undifferentiated cultured cells, such as preadipocytes from brown fat and neonatal astroglial cells (14, 15). In addition, T_3 and retinoic acid stimulate D3 expression in these cells and *in vivo* (16–18). These interesting patterns of expression and regulation of D3 during development suggest an important role for this enzyme in limiting the exposure of fetal tissues to maternal thyroid hormones. Such a role would be of considerable importance given the observed detrimental effects during development of exposure to excessive levels of thyroid hormone (19, 20).

To further characterize the structural and functional features of D3, we have isolated a mouse genomic DNA clone that contains the gene (*Dio3*) for this enzyme. We report herein the initial characterization of *Dio3*, including its promoter region.

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Address all correspondence and requests for reprints to: Dr. Donald L. St. Germain, Dartmouth Medical School, One Medical Center Drive, Lebanon, New Hampshire 03756.

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† Recipient of research fellowships from NATO and the Comision Interministerial de Ciencia y Tecnologia, Spain.

‡ Current address: Cornell/Rockefeller/Memorial Sloan-Kettering Tri-Institutional M.D./Ph.D. Program, New York, New York 10021.

Materials and Methods

Isolation of Dio3 clones

A murine (129/SvJ strain) genomic library constructed in the λ Dash II vector (Stratagene, La Jolla, CA) was screened by plaque hybridization using the rat NS27-1 D3 cDNA (6) labeled with [^{32}P]deoxy-CTP (ICN Biochemicals, Inc., Costa Mesa, CA) as a probe. Radiolabeling of cDNA probes for library screening and for Southern and Northern blotting was performed using the oligolabeling kit from Pharmacia Biotech (Piscataway, NJ), and the probe was purified through NucTrap columns (Stratagene).

Restriction mapping, subcloning, and sequencing

Restriction mapping was performed by digestion of the *Dio3* clone with various restriction enzymes followed by Southern blotting using standard procedures and part or all of the full-length rat NS43-1 D3 cDNA as a probe. Genomic fragments were subcloned into the following vectors: pBluescript SK (Stratagene), pGL3 (Promega Corp., Madison, WI), and pXP2 (21).

Sequencing was performed using vector- and gene-specific primers and an automated sequencing system with fluorescent dye terminators (PE Applied Biosystems, Foster City, CA).

Cell culture and transient transfections

COS-7 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as previously described (9). The XTC-2 and XL-2 cell lines, derived from *Xenopus laevis* adult carcass and premetamorphic tadpoles, respectively, were provided by Dr. J. Tata (National Institute for Medical Research, The Ridgeway, London, UK) and cultured in 0.6 × L-15 (Leibovitz) medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Life Technologies, Gaithersburg, MD). Primary cultures of rat preadipocytes were obtained as previously described (16) from 18-day-old Sprague-Dawley rats, seeded at approximately 600 cells/cm^2, and cultured in high glucose DMEM supplemented with 10% FBS, 15 μM ascorbic acid, and gentamicin (50 μg/ml).

XTC-2 and XL-2 cell lines were plated at 3×10^5 cells/60-mm culture dish the day before transfection, whereas primary brown fat preadipocytes were plated at 1.5×10^4 cells/60-mm dish 4 days before transfection. All cell types were transfected with 9 μg DNA using the calcium phosphate method using a kit from Clontech (Palo Alto, CA). Cells were cotransfected with 5 μg β-galactosidase expression vector (pCH110, Pharmacia Biotech) to correct for transfection efficiency. After overnight exposure to the DNA precipitate, the cells were cultured for 48 h before harvesting. Luciferase and β-galactosidase activities were determined in cell lysates using assay kits from Promega Corp. Light emission was quantified using an EG & G Berthold microplate luminometer LB 96V (Wallac, Gaitherburg, MD).

Northern blotting

RNA was prepared, and Northern blotting was performed as previously described (14). In brief, total RNA was obtained from rat brown fat preadipocytes and from mouse placentas and fetal livers (17 days of embryonic life) by a guanidinium chloride-based method. Polyadenylated [poly(A)$^+$] RNA was prepared by standard oligo(deoxythymidine)-cellulose affinity chromatography. RNA samples were run in a denaturing 1% agarose gel and blotted to a nylon membrane (Schleicher & Schuell, Inc., Keene, NH). The filter was hybridized for 24 h at 42 C with a radiolabeled restriction fragment from the mouse D3 genomic clone (*Xho*I/*Bam*HI) that included the coding region and the 5′-UT region of the *Dio3*. The filter was then washed twice for 30 min each time at 60 C with 0.1% SDS and 0.1 × SSC and autoradiographed.

Primer extension

Primer extension was performed according to the procedures of Sambrook *et al.* (22) using an antisense primer (mST75) that spans the translational start site (5′-CAG AGA GCG GAG CAT GGT GG-3′, where the *underlined* CAT triplet represents the position of the ATG start codon). Briefly, the mST75 primer was labeled with [γ-^{32}P]ATP (ICN Biochemicals, Inc.) using the primer extension kit from Promega Corp., then denatured and allowed to anneal to 1 μg mouse placental or fetal liver poly(A)$^+$ RNA overnight at 60 C in the presence of 80% formamide, 40 mM PIPES (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. After ethanol precipitation, RT was carried out for 1 h using 40 U AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN). Reaction products were extracted with phenol, precipitated with ethanol, and run on an 8% polyacrylamide 7 M urea gel. To allow an accurate determination of product size, sequencing reactions were also performed using the same primer and were loaded onto the same gel in adjacent lanes. The sequencing reactions were performed using a 1.5-kb *Bam*HI/*Kpn*I fragment of the D3 genomic clone (that had been subcloned into pBluescript) as a template, [^{35}S]deoxy-ATP (ICN Biochemicals, Inc.) as a labeling reagent, and the Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, OH).

Results

Isolation and sequence analysis of a Dio3 clone

We screened a mouse liver genomic library with a rat D3 cDNA probe and isolated a positive clone containing a 12-kb DNA insert. Restriction analysis followed by Southern blotting using the full length or various fragments of the rat D3 cDNA allowed us to deduce the structural features of *Dio3*, as shown in Fig. 1. An example of this analysis is shown in Fig. 2, where the genomic clone in the λ Dash II vector has been digested with *Kpn*I and *Bam*HI. A diagram of the location of these restriction sites in the isolated clone is shown in Fig. 2A. As shown in lane 1 of Fig. 2B, digestion results in fragments of approximately 17.5, 10.5, and 1.5 kb derived largely from the vector arms and five fragments of 4.2, 3.2, 2.0, 1.5, and 0.7 kb derived from the *Dio3* gene insert. These fragments were then subjected to Southern analysis, using as probes the fragments of the rat D3 cDNA shown in Fig. 2A. As shown in lane 2 of Fig. 2B, a portion of the rat D3 cDNA corresponding to the 5′-UT region plus 178 bp of the adjoining coding region (probe A) hybridized only to the 1.5 kb restriction fragment. A similar result was observed using probe B, which is derived entirely from the rat D3 coding region (lane 3). Probe C encompassing the entire 3′-UT region of the rat cDNA hybridized to both the 1.5- and 4.2-kb fragments (lane 4).

These results suggested that the entire length of the D3 mRNA that is expressed in the placenta and skin was encoded within the segment of the *Dio3* gene that encompassed

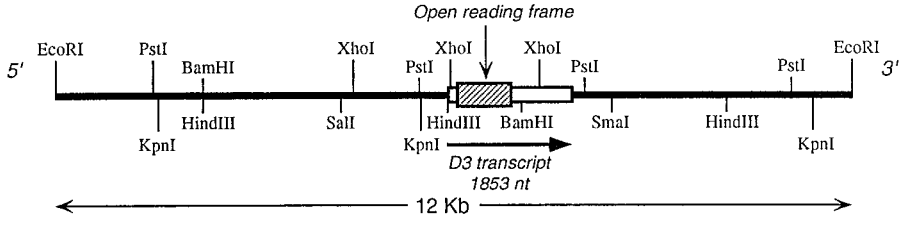

FIG. 1. Restriction map and structural features of the 12-kb mouse *Dio3* genomic fragment. The single exon is shown with the open reading frame indicated by the *hatched area*.

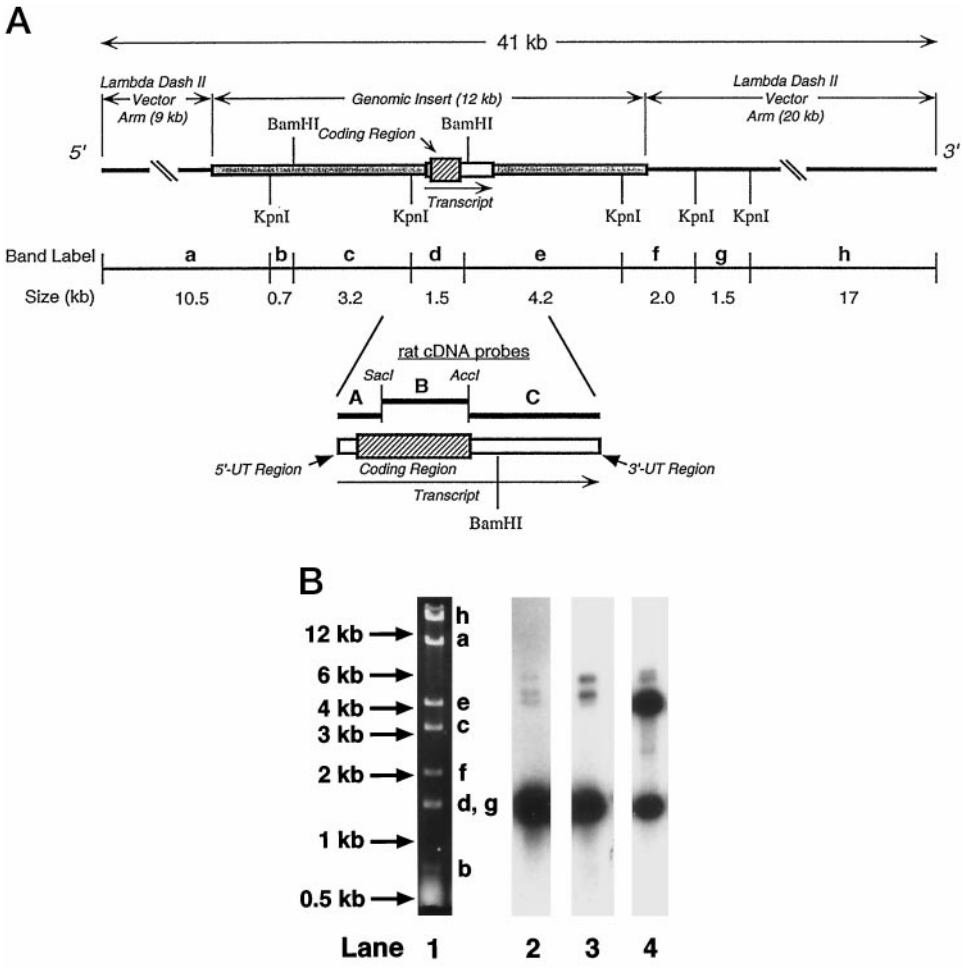

FIG. 2. A, A diagram of the *Kpn*I and *Bam*HI restriction sites in the *Dio3* genomic clone (including the λ Dash II vector). Lettered fragments correspond to the bands in lane 1 of B. Shown in the *lower portion* of this figure is a map illustrating the positions of the rat D3 cDNA probes used for the Southern analysis. B, Southern analysis of *Dio3* *Kpn*I/*Bam*HI restriction fragments probed with various portions of the rat D3 cDNA. Lane 1, Ethidium bromide-stained restriction fragments after agarose gel electrophoresis. Fragments of 17 and 10.5 kb represent the phage vector arms plus, in the case of the smaller band, terminal sequences from the genomic insert. Bands of 4.2, 3.2, 2.0, and 0.7 kb are derived from the genomic insert. The 1.5-kb band represents two fragments of the same size, one that includes promoter and exonic sequences of the *Dio3* genomic insert (band d) and one from the vector arm (band g). Lane 2, Southern blot of restriction fragments probed with a portion of the rat D3 cDNA containing the 5′-UT region and 178 bp of the coding region (probe A). Lane 3, Southern blot probed with a portion of the coding region of the rat D3 cDNA (probe B). Lane 4, Southern blot probed with the 3′-UT region of the rat D3 cDNA (probe C).

the 1.5- and 4.2-kb restriction fragments. This was confirmed by subcloning the 2.5-kb *Hin*dIII/*Sma*I restriction fragment that contained the D3 cDNA sequences into pBluescript. Sequencing of this fragment and comparing it to the rat neonatal skin D3 cDNA sequence demonstrated that the murine D3 gene contains a single exon, 1853 nucleotides in length, that encodes the placenta/skin mRNA.

The predicted coding region sequence of the mouse *Dio3* (Fig. 3) shows high homology to the rat (6) and human (7) D3 cDNAs at both the nucleotide (96% rat and 87% human identity) and the predicted amino acid (99% rat and 96% human identity) levels. This includes an in-frame TGA triplet that codes for selenocysteine. A high degree of nucleotide identity (94%) is also noted in the SECIS-containing portion of the 3′-UT region and in the overall 3′-UT region (86%), including the presence of a consensus polyadenylation sequence at the 3′-end. As expected, lesser degrees of homology are observed when comparing the mouse D3 protein to those of lower species; amino acid identities with *X. laevis* (5) and chicken (23) D3 are 55% and 58%, respectively.

Northern analysis of RNA extracted from mouse placenta and probed with a 1.2-kb *Hin*dIII/*Bam*HI genomic fragment from the mouse coding region (see Fig. 1) demonstrated a D3 transcript similar in size (2.2 kb) to that previously described in the rat (Fig. 4). As a positive control, RNA from epidermal growth factor-stimulated rat brown preadipocytes was included on this blot (14).

Defining the promoter region of the mouse D3 gene

A sequence comparison of the putative 5′-UT regions of the mouse, rat (6), and human D3 (7) cDNAs demonstrated that the first 64 nucleotides 5′ of the ATG translational start site are identical. Thereafter, however, the sequences diverge to some extent, suggesting that the transcriptional start site could be in this region. To more precisely determine its location, primer extension experiments were performed using mouse placenta and liver poly(A)$^+$ as a template and a labeled oligonucleotide primer whose 3′-end was immediately upstream of the translational start site. Analysis of the reaction products showed a predominant band approximately 75 bp in size and a lesser band of 92 bp, with placenta and liver poly(A)$^+$ RNA resulting in the same pattern of bands (Fig. 5). Additional primer extension experiments using a variety of denaturing and annealing conditions were performed, and in all cases both reaction products were obtained, although their relative abundances varied. In most reactions, the larger reaction product was more abundant. Of note, this region of the genomic sequence is GC rich, which could have accounted for these varying results. Given the

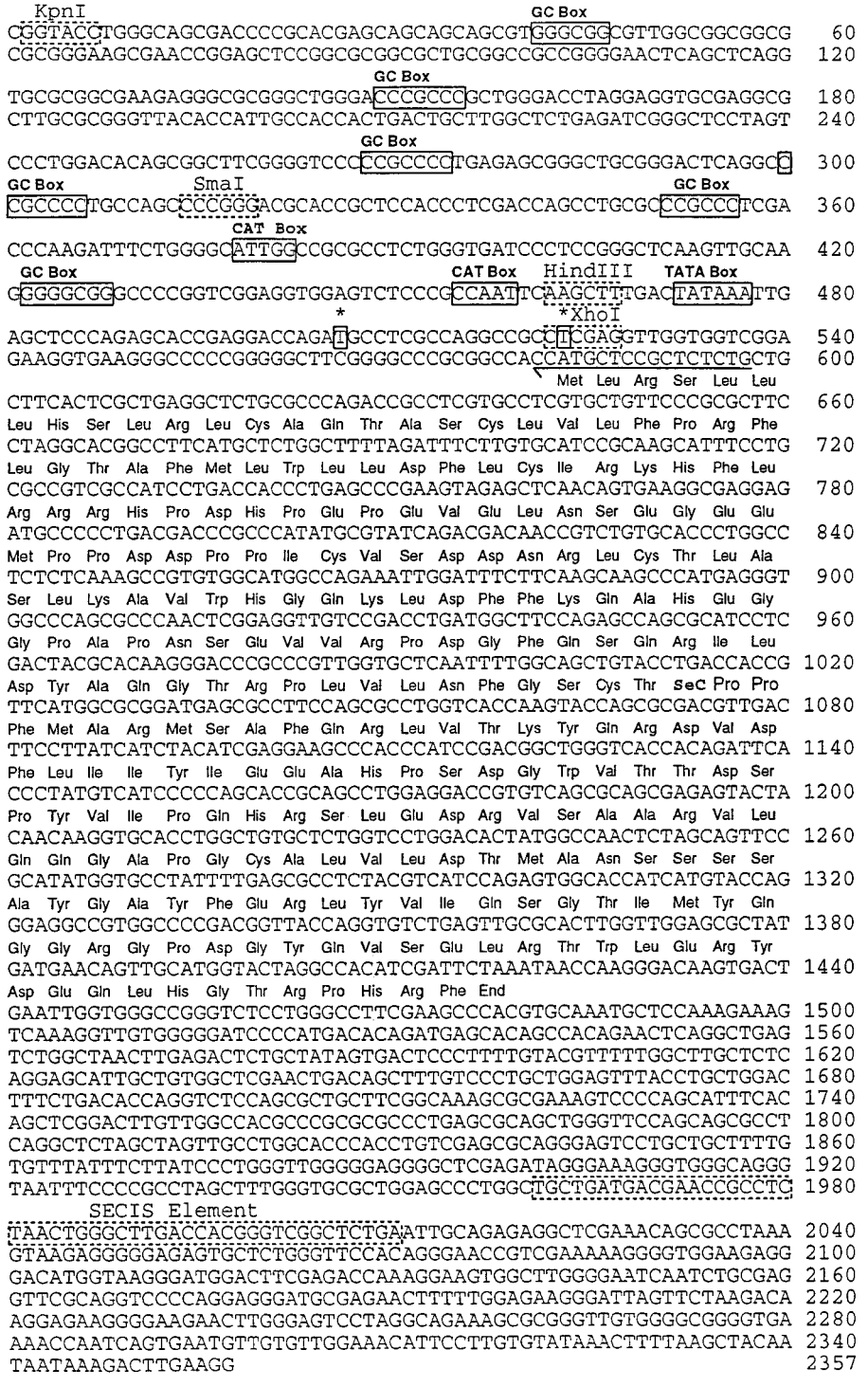

FIG. 3. Nucleotide sequence of the mouse *Dio3* exon and approximately 500 nucleotides of the immediate upstream region. The transcriptional start sites, as determined by primer extension analysis, are indicated by *asterisks above the boxed nucleotides* at positions 506 and 523. The ATG translational start codon is at position 583, and the TGA selenocysteine codon is at position 1012. Various consensus promoter elements present on either strand of the DNA are shown in *solid boxes*, and restriction enzyme sites are *outlined in dotted boxes*. The conserved SECIS element extends from nucleotides 1961–2010. The *underlined* nucleotides at 578–597 represent the location of the mST75 antisense oligonucleotide used in the primer extension reactions.

location of the mST75 primer used in the primer extension studies, the sizes of the reaction products indicates that the transcriptional start sites are 77 and 60 nucleotides upstream of the ATG translational start codon.

The two putative transcriptional start sites, as defined by primer extension, are located 29 and 46 bp downstream of a consensus TATA box (Fig. 3). Upstream of this element are putative CAAT and GC box sequences on both strands of the DNA, consistent with this being the promoter region of the D3 gene. Notably, the sequence in this region is GC rich, a feature that is common to many gene promoters. To determine whether this region could serve as a functional promoter, a 526-bp *Kpn*I/*Xho*I fragment, located immediately upstream of the most 3′-putative transcriptional start site (see Fig. 3), was subcloned in front of a luciferase reporter gene in the pXP2 vector. This construct was transfected into COS-7 cells, *Xenopus* XTC-2 and XL-2 cells, and primary preadipocytes derived from rat brown fat. Luciferase activity

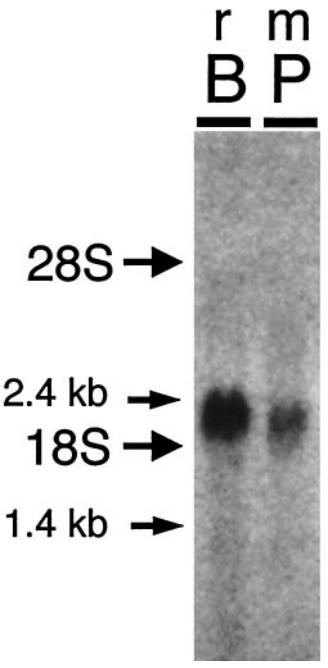

FIG. 4. Northern analysis of total RNA (20 μg) from cultured rat brown preadipocytes (lane rB) and mouse placenta [2 μg poly(A)$^+$; lane mP] probed with a portion of the coding region of the mouse D3 genomic clone.

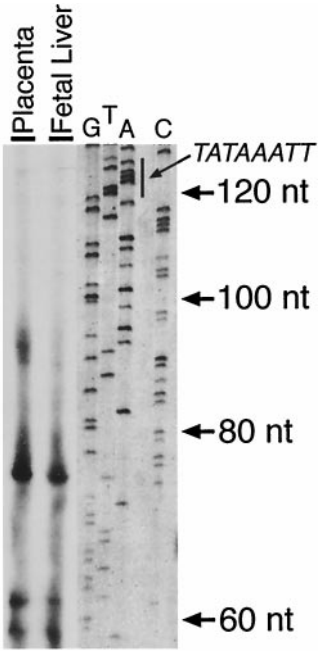

FIG. 5. Primer extension reaction using mouse placental or fetal mouse liver poly(A)$^+$ RNA as template and the mST75 antisense oligonucleotide shown in Fig. 3 as primer. Sequencing reactions prepared with the same primer are shown for size comparison. The location of the putative TATA box is indicated.

was then determined in cell lysates. The pXP2 plasmid containing only the luciferase gene was used as a negative control, and the plasmid pCH110 in which the β-galactosidase gene is driven by the simian virus 40 early promoter was used as a control for transfection efficiency. As shown in Fig.

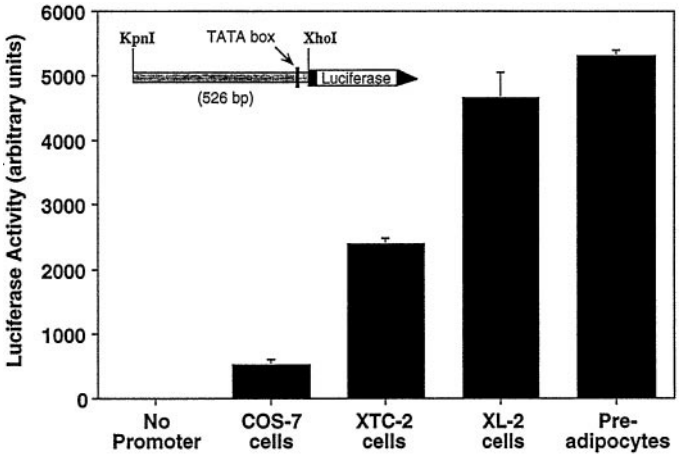

FIG. 6. Analysis of promoter function of a 526-bp *Kpn*I/*Xho*I *Dio3* fragment. The genomic fragment was cloned into the pXP2 vector so as to drive luciferase activity, and this construct was then transfected into COS-7, XTC-2, or XL-2 cells or into primary cultures of rat preadipocytes derived from neonatal brown fat. Luciferase activity was determined in cell lysates 48 h after transfection. Cells were also cotransfected with a β-galactosidase expression plasmid for correction of transfection efficiency. The data in the first column, labeled no promoter, are derived from transfection of the empty pXP2 vector into COS-7 cells and are representative of the values obtained when this vector was transfected into the other cell lines. The values shown are the mean ± SD of triplicate dishes from a single experiment. Similar findings were noted in a replicate experiment.

6, luciferase activity was minimal in cells transfected with the pXP2 control plasmid. However, in cells transfected with the construct containing the *Kpn*I/*Xho*I D3 genomic fragment, luciferase activity was increased 500-5000 times, demonstrating the presence of strong promoter elements within this region. Activity was highest in the preadipocyte and XL-2 cells, with considerably less activity observed in COS-7 cells.

To further characterize the key elements responsible for the basal activity of the D3 promoter, we subcloned a 214-bp *Sma*I/*Xho*I restriction fragment or the above-described 526-bp *Kpn*I/*Xho*I fragment into the pGL3 luciferase reporter vector (Promega Corp.). Both of these constructs included the putative TATA box at their 3′-ends. In addition, two similar constructs that lacked the putative TATA box were prepared by using the *Hin*dIII restriction site as the 3′-end of the fragment. These four constructs, in addition to the pGL3 vector containing luciferase alone as the negative control, were transfected individually into XTC-2 cells along with the β-galactosidase reporter plasmid, and luciferase activity was then determined (Fig. 7). Both the 526- and 214-bp genomic fragments, which contain the intact TATA element, demonstrated strong promoter activity. In contrast, deletion from both constructs of the TATA box largely eliminated promoter activity.

Discussion

The D3 is unique among the selenodeiodinases in that it catalyzes exclusively inner ring deiodination and is most highly expressed in mammals during early fetal development and in the placenta (1). This expression pattern in effect isolates the fetal thyroid axis from that of the mother and appears designed to maintain T_4 and T_3 levels relatively low

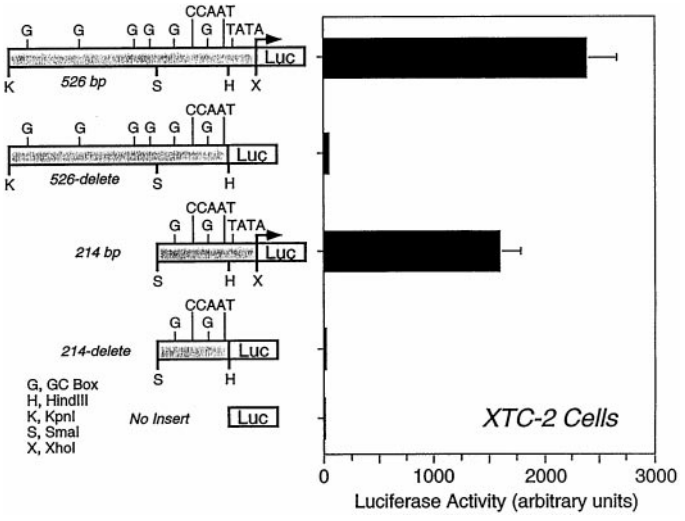

FIG. 7. Effect of deletion of the putative TATA box on promoter function of two *Dio3* genomic fragments. The 526-bp *Kpn*I/*Xho*I fragment and a 214-bp *Sma*I/*Xho*I fragment were subcloned into the pGL3 luciferase reporter vector. Vectors were also prepared using the *Kpn*I/*Hin*dIII (526-delete) and the *Sma*I/*Hin*dIII (214-delete) fragments, both of which exclude the putative TATA box from the construct. These constructs were then transfected into XTC-2 cells along with a β-galactosidase expression plasmid to correct for transfection efficiency. Luciferase and β-galactosidase activity were determined in cell sonicates 48 h after transfection. The pGL3 vector lacking a *Dio3* genomic fragment (No Insert) served as a control. The values shown are the mean ± SD of triplicate dishes from a single experiment.

in the early stages of development. To the extent that the level of the D3 is regulated by transcriptional processes, its promoter region must contain control elements that dictate its expression in both a tissue-specific and developmental stage-related manner. In addition, the D3 appears to be regulated by a number of hormones (16–18) and growth factors (14, 15) that probably influence its expression patterns during development and in the adult. In the present report, we describe the isolation and initial characterization of the mouse *Dio3.* The availability of this genetic material should provide important insights into the developmental regulation of thyroid hormone metabolism and action.

The major features of the mouse *Dio3* gene are 1) that its sequence in both the coding and 3′-UT regions has been highly conserved among diverse species; 2) that a single exon encodes the full length of the mRNA species expressed in the placenta and skin; and 3) that its promoter region, which is located within 78 bp of the translational start site, is a C+G-enriched (80%) region containing a TATA box that is critical for its function as well as CAAT and GC boxes. Initial characterization of a human P1 clone containing the *DIO3* gene indicates that these structural features have been conserved in this species (Hernandez and St. Germain, unpublished studies). In addition, sequence analysis demonstrates 80% nucleotide identity between the first 500 bp of the mouse and human promoter regions. This high degree of structural conservation may attest to the importance of the developmental role of this enzyme throughout the vertebrate kingdom.

Our identification of the promoter region of the mouse *Dio3* gene is based on 1) the results of Northern analysis, which identified the size of the mouse D3 mRNA to be approximately 2.2 kb; 2) the results of primer extension experiments, which place the start site(s) of transcription at 77 and/or 60 bp upstream of the translational initiation codon; 3) the structural features of the putative upstream regulatory region, which contains several consensus basal promoter elements; and 4) the ability of this region to drive high levels of expression of a heterologous gene construct in a number of cell culture systems. Thus, a 526-bp region upstream of the start of transcription induces high levels of luciferase reporter activity in *Xenopus* XTC-2 and XL-2 cell lines and in primary cultures of preadipocytes from rat brown fat. These cells express endogenous D3 activity or can be induced to do so by treatment with various hormones or growth factors. In contrast, promoter activity, although present, is greatly attenuated in COS-7 cells, which do not express D3 activity in their native state. These results suggest that tissue-specific regulatory elements may be present within the region of the D3 promoter that was tested.

Although D3 expression is regulated by thyroid hormones and retinoic acid *in vivo* (24, 25) and in cell culture systems (16–18), no consensus sequences for thyroid hormone or retinoic acid response elements are found by inspection of the first 500 bp of the 5′-flanking region. Growth factors and phorbol esters have also been noted to stimulate D3 activity and mRNA levels, but only limited information is available on the transcription factors that mediate these transduction pathways. Thus, further studies that will probably require the characterization of additional upstream regulatory sequences will be needed to identify the elements involved in conferring responsiveness to these factors.

This first report of the structure of a mammalian D3 gene demonstrates that the coding region and known 5′-UT and 3′-UT regions, which include the SECIS element, are contained within a single exon encompassing 1853 nucleotides. Thus, D3 may be a member of a relatively small group of single exon genes. Such genes comprise only 6% of the typical mammalian genome, although they are more common in lower species (26). Larger D3 transcripts have been observed in the rat brain (6), however, suggesting that alternative polyadenylation sites or additional exons may be present in the *Dio3* gene.

The D3 genomic structure defined herein contrasts with the recently described structures of the human *DIO1* (27) and *DIO2* (28), which contain four and two exons, respectively. As it is reasonable to hypothesize that the three deiodinase genes have evolved from a common ancestor, this variability in genomic structure is of considerable interest. Further comparative studies of the genomic structures of this family of genes in different species may provide important clues concerning their evolutionary development.

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