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Biochemistry, Cellular and Molecular Biology, and Physiological Roles of the Iodothyronine Selenodeiodinases

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The goal of this review is to place the exciting advances that have occurred in our understanding of the molecular biology of the types 1, 2, and 3 (D1, D2, and D3, respectively) iodothyronine deiodinases into a biochemical and physiological context. We review new data regarding the mechanism of selenoprotein synthesis, the molecular and cellular biological properties of the individual deiodinases, including gene structure, mRNA and protein characteristics, tissue distribution, subcellular localization and topology, enzymatic properties, structure-activity relationships, and regulation of synthesis, inactivation, and degradation. These provide the background for a discussion of their role in thyroid physiology in humans and other vertebrates, including evidence that D2 plays a significant role in human plasma T_3 production. We discuss the pathological role of D3 overexpression causing "consumptive hypothyroidism" as well as our current understanding of the pathophysiology of iodothyronine deiodination during illness and amiodarone therapy. Finally, we review the new insights from analysis of mice with targeted disruption of the *Dio2* gene and overexpression of D2 in the myocardium. (*Endocrine Reviews* 23: 38–89, 2002)

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Abbreviations: aFGF, Acidic fibroblast growth factor; Ala, alanine; BAT, brown adipose tissue; bFGF, basic fibroblast growth factor; BiP, endoplasmic reticulum resident binding protein; BrAc, *N*-bromoacetyl; CMZ, ciliary marginal zone; CNS, central nervous system; CRE, cAMP responsive element; Cys, cysteine; D1, D2, D3, types 1, 2, and 3, respectively, iodothyronine deiodinases; Dkk, Dickkopf proteins; DTT, dithiothreitol; EFsec, specificity for selenocysteyl-tRNA; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK, extracellular receptor kinase; FR, flanking region; GPX, glutathione peroxidase; GRP78, glucose-regulated protein 78; GTG, gold thioglucose; HCN2, hyperpolarization-activated cyclic nucleotide-gated channel 2; His, histidine; IRD, inner-ring deiodinations; K_m, Michaelis-Menten constant; MCR, metabolic clearance rate; MHC, myosin heavy chains; NE, norepinephrine; NF-κB, nuclear factor κB; nt, nucleotide; ORD, outer-ring deiodinations; PTU, 6-*n*-propyl-2-thiouracil; RACE, rapid amplification of cDNA ends; SBP2, SECIS binding protein 2; Se, selenium Sec, selenocysteine; SECIS, Sec insertion sequence; selA, Sec synthase; selB, elongation factor with mRNA stem-loop binding activity; selC, tRNA [Ser]Sec; selD, selenophosphate synthase; SNS, sympathetic nervous system; SRC, steroid receptor coactivator; T_2S, diiodothyronine sulfate; T_3S, T_3 sulfate; TRE, thyroid hormone response element; TSS, transcription start site; TTF-1, thyroid transcription factor-1; Ub, ubiquitin; UCP1, uncoupling protein-1; UTR, untranslated region; V_{max}, maximum velocity.

I. Introduction and Historical Review

IT IS NOW 50 yr since the publication of the first studies demonstrating the appearance of an unknown labeled compound in the tissues of animals and humans given

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[^{131}I]T_4, which was eventually identified as T_3 by Gross and Pitt-Rivers (1). Because T_3, not T_4, is the TR-bound hormone, outer ring (5′) deiodination can be viewed as the first step in the activation of the thyroid prohormone T_4. T_4 5′ monodeiodination supplies at least 80% of T_3 in humans (Fig. 1 and Ref. 2). Twenty years passed before the development of assays for quantitation of T_3 in human serum reawakened interest in this activation step (3). Work over the subsequent two decades consisted primarily of documenting the presence of two different enzyme activities that catalyzed T_4-to-T_3 conversion, the types 1 and 2 iodothyronine deiodinases (D1 and D2, respectively), and the identification of an inner ring deiodinase, which can inactivate T_4 or T_3 (4, 5). D1 and D2 were first distinguished by the presence (D1) or absence (D2) of sensitivity to inhibition by 6-*n*-propyl-2-thiouracil [PTU (6–10)]. It is important to recognize that, due to the free rotation of the phenolic (outer) ring in the iodothyronine molecules, monodeiodination at the 5 or 3 positions of the tyrosyl ring are equivalent inner-ring deiodinations (IRD), and those of the 3′ or 5′ positions (phenolic ring) are equivalent outer-ring deiodinations (ORD). In this review we will refer to ORD and IRD as 5′ and 5, respectively (Fig. 1).

Cloning of the rat D1 cDNA identified a selenocysteine (Sec) codon, UGA, in the catalytic site of D1 (11), explaining the significant decrease in D1 activity reported in selenium (Se)-deficient rats (12–14). Subsequent studies led to the cloning of type 3 iodothyronine deiodinase (D3), which was first recognized as a highly T_3-responsive cDNA with similarity to D1 in *Xenopus laevis* tadpoles (15). Most recently, the cDNA encoding D2 from *Rana catesbeiana* was cloned, and soon thereafter, the coding regions of the rat and human D2 proteins were identified (16, 17). D2 and D3 also contain Sec as part of a highly similar active center in all species cloned to date, illustrating the importance of this rare amino acid in the deiodination reaction (Fig. 2). The complete cDNA sequences of the human, chicken, and mouse D2 cDNAs containing the critical mRNA structures required for Sec incorporation (see *Section III.B*) were subsequently identified (18–20).

The cotranslational incorporation of Sec into the deiodinases and other selenoproteins presents significant problems for the cell, which must recognize the UGA as a Sec codon rather than a STOP translation signal. The cloning of D1 led to the identification of the eukaryotic Sec insertion sequence (SECIS) element as a stem-loop structure in the 3′ untranslated regions (UTR) of the D1 and glutathione peroxidase mRNAs. The SECIS element is the signal that recodes the in-frame UGA from a STOP to a Sec codon (21). An additional 10 yr were required for the essential components of the eukaryotic selenoprotein synthesis machinery to be identified (22, 23). Lastly, whereas the general features of the physiological role of these deiodinases and the metabolic transformations that they catalyze have been appreciated for many years, the preliminary results of the first targeted disruption of D2 were reported in October 2000 at the 12th International Thyroid Congress (24).

The goal of this review is to place the exciting advances that have occurred in our understanding of the molecular biology of the iodothyronine deiodinases into a biochemical and physiological context. The reader is referred to several earlier reviews for a more detailed scientific background of concepts underlying much of the work to be discussed below (25–29). Although we will focus on new insights, these will be placed in the context of previous knowledge to allow presentation of a coherent picture of the role of the deiodinases in thyroid physiology. After a discussion of the mechanism of selenoprotein synthesis, we will review the specific molecular and cellular biological properties of the individual deiodinases. These provide the background for a discussion of their role in thyroid physiology and pathophysiology.

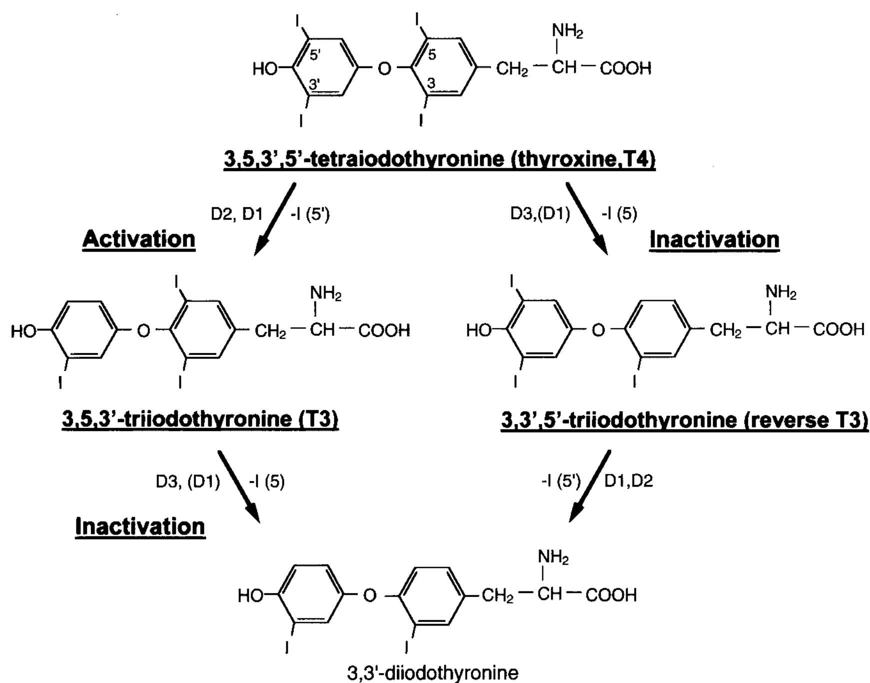

FIG. 1. Structures and interrelationships between the principal iodothyronines activated or inactivated by the selenodeiodinases.

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Whenever possible, we have focused the discussion on the biological role of these enzymes in human physiology.

II. The Synthesis of Selenoproteins

Of the avenues of research opened through identification of the first iodothyronine deiodinase cDNA, several could have been anticipated, but some were quite surprising at the time. Availability of the rat D1 cDNA provided the first primary sequence of one of the deiodinase enzymes, the analysis of which revealed several features shown to be important for structure and activity. The most unusual of these was the presence of the rare amino acid, Sec, in the active site, encoded by UGA (11). Its critical role in the function of the enzyme was ascertained through characterization of the kinetic properties of the wild-type selenoenzyme and the corresponding cysteine (Cys) mutant (30). The D1 sequence ultimately provided the information needed for identifying cDNAs encoding the type 2 and 3 deiodinases, which are also selenoenzymes (Fig. 2). Thus, a major unanticipated avenue of research had its beginnings with the identification of Sec in the active site of rat D1–the investigation of the requirements for and mechanism of Sec incorporation in eukaryotes.

HumanD1	:	RPLVLNFGSCT*PSF
RatD1	:	RPLVLNFGSCT*PSF
MouseD1	:	RPLVLNFGSCT*PSF
DogD1	:	RPLVLNFGSCT*PSF
ChickenD1	:	RPLILNFGSCT*PSF
TilapiaD1	:	RPLVLSFGSCT*PPF
Consensus		RPLvLnFGSCT*PsF
HumanD3	:	RPLVLNFGSCT*PPF
RatD3	:	RPLVLNFGSCT*PPF
ChickenD3	:	RPLILNFGSCT*PPF
XenopusD3	:	RPLVVNFGSCT*PPF
RanaD3	:	RPLVLNFGSCT*PPF
TilapiaD3	:	RPLILNFGSCS*PPF
Consensus		RPLvlNFGSCt*PPF
HumanD2	:	RPLVVNFGSAT*PPF
RatD2	:	RPLVVNFGSAT*PPF
MouseD2	:	RPLVVNFGSAT*PPF
ChickenD2	:	RPLVVNFGSAT*PPF
RanaD2	:	RPLVVNFGSAT*PPF
FundulusD2	:	RPLVVNFGSAT*PPF
Consensus		RPLVVNFGSAT*PPF

FIG. 2. Amino-acid sequence homology of the active catalytic centers of the deduced amino acid sequences of the three classes of selenodeiodinases. The high conservation of residues within the active center argues for similarities in the deiodination mechanism among the three enzymes. An *asterisk* indicates a Sec.

A. Recoding UGA from STOP to selenocysteine (Sec)

1. Identification of the Sec insertion element (SECIS) element. Type 1 deiodinase was only the second eukaryotic mRNA shown to encode a selenoprotein, the first being classical glutathione peroxidase (GPX). However, little was known about the mechanism of synthesis of selenoproteins in eukaryotes. Several prokaryotic selenoprotein cDNAs had been sequenced, and using these cDNAs in biochemical and genetic studies, the *cis*-acting sequences and *trans*-acting factors required for Sec incorporation in prokaryotes had been elucidated (31, 32). The *cis*-acting sequences consist of the Sec codon itself, UGA, and a specific RNA stem loop immediately downstream of the UGA codon. UGA is recognized in the vast majority of mRNAs as a STOP codon. Only in the presence of the stem-loop structure and *trans*-acting factors are UGA codons "recoded" to specify Sec instead.

The *trans*-acting factors identified in bacteria are encoded by genes designated Sec synthase (selA), elongation factor with mRNA stem-loop binding activity (selB), tRNA [Ser]Sec (selC), and selenophosphate synthase [selD (Table 1 and Refs. 31 and 33–37)]. Both selA and selD encode enzymes required for Sec biosynthesis, and selC encodes a unique tRNA possessing an anticodon complementary to UGA and a secondary structure that differs from all other tRNAs. selB encodes a protein with two distinct functional domains. The first, an elongation factor domain, recognizes selenocysteyl-tRNA via its unique structure and amino acid and delivers the tRNA to the ribosome. The second domain, a C-terminal extension, specifically binds the RNA stem loop downstream of the UGA codon in prokaryotic selenoprotein mRNAs. Thus, recruitment of the elongation factor via the RNA stem loop results in recoding of only the immediately adjacent UGA.

A Sec-specific tRNA had previously been identified in eukaryotes (38, 39), as had the UGA encoding Sec in the GPX sequence (40). But it was quickly appreciated that the conserved secondary structures adjacent to the UGA Sec codons in the coding regions of prokaryotic selenoprotein mRNAs were absent in the D1 and GPX mRNAs. Deletion mapping studies performed during the characterization of the D1 cDNA provided the first clue of a major difference between the prokaryotic and eukaryotic mechanisms for selenoprotein synthesis—sequences in the 3′ UTR were required for expression of a functional enzyme from a TGA-containing D1 cDNA construct, but not from a mutant differing solely

TABLE 1. Genes required for selenoprotein synthesis

Prokaryotes		Eukaryotes	
selA	SEC	selA	SEC
selB	Selenocysteyl-tRNA-specific elongation factor with mRNA stem-loop binding activity	Efsec	Selenocysteyl-tRNA-specific elongation factor
		SBP2	SECIS-binding factor that interacts with EFsec
selC	tRNA [Ser]Sec	selC	tRNA [Ser]Sec
selD	Selenophosphate synthetase	SPS1	Selenophosphate synthetase (nonselenoenzyme)
		SPS2	Selenophosphate synthetase (selenoenzyme)

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by the substitution of a TGT-Cys codon (21, 30). This clearly demonstrated that the 3′-UTR sequences were required for translation of the Sec codon. It contrasts with the UGA-proximal coding region location of the corresponding prokaryotic sequences. Further deletion analysis more precisely defined the region required, and computer folding algorithms applied to this narrowly defined region predicted the formation of a stable hairpin or stem-loop structure (Fig. 3). Examination of the GPX sequence revealed the potential of its 3′ UTR to form a similar stem loop. In addition, both the D1 and GPX stem loops contain three short regions of conserved nucleotide sequence. The property of these two elements to function interchangeably in conferring D1 expression led to the concept of the stem loop structure in the 3′ UTR as necessary and sufficient for conferring Sec incorporation at UGA codons and as a feature whose presence would be a hallmark of selenoprotein mRNAs. It was thus termed the SECIS element (21).

2. SECIS sequence, structure, and spacing requirements. Subsequent studies over the ensuing years focused on detailed characterization of the sequence, structural, and spacing constraints of eukaryotic SECIS elements. With the subsequent identification of new selenoprotein sequences in eukaryotic species ranging from protozoans to humans, for a current total of over 20 in vertebrates, the basic sequence and structural features of the first SECIS elements were found to be conserved in every case (41–45). After the initial studies with the D1 and GPX SECIS elements, the D1 activity assay also allowed comparison of the relative activities of SECIS elements from different selenoproteins and of the effects of mutations introduced into these elements (41, 42, 46, 47). Thus, the standard assay for SECIS function became the generation of constructs containing the D1 coding region

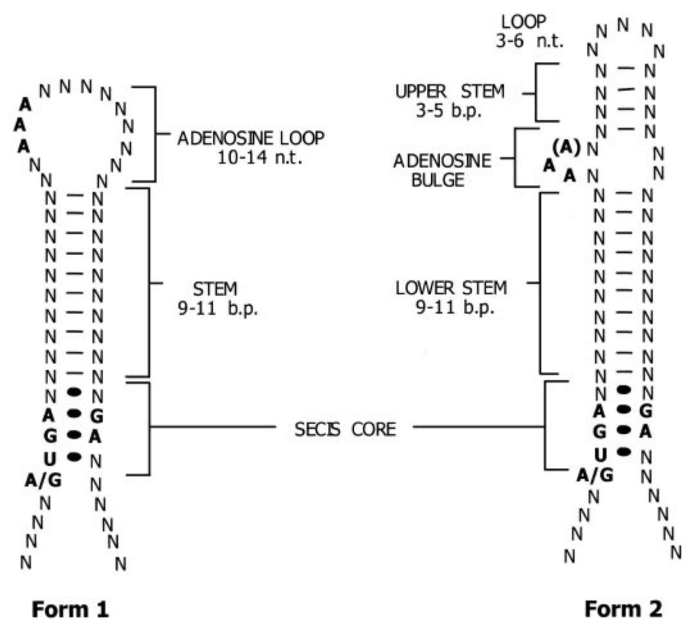

FIG. 3. Consensus SECIS element structures. Conserved sequence and structural features include the SECIS core nucleotides, A/GUGA and GA, the stem length, and conserved adenosines in a terminal loop (Form 1) or bulge (Form 2). *Lines* indicate Watson-Crick base pairs, and *filled ovals* designate non-Watson-Crick pairing.

linked to heterologous SECIS elements, followed by transient transfection of these constructs and quantitation of the resulting D1 enzyme activity. Comparison of numerous SECIS elements in this way revealed that the activities of most naturally occurring elements fall within a relatively narrow range, with a few exceptions (23). In addition, this assay allowed more precise definition of the required nucleotides and secondary structural characteristics. For example, the sequences of the stems are not constrained, provided base pairing is maintained (42, 46). In contrast, the conserved nucleotides, A/GUGA at the 5′ base of the stem, AA in the hairpin loop, and GA at the 3′ base of the stem (42, 46, 48), were shown to be critical for function (Fig. 3). The minimal sequence required for SECIS function was defined, the boundaries of which correspond precisely with the conserved 5′ A/GUGA and 3′ GA sequences (46). This region was subsequently shown to form non-Watson-Crick base pairs: purine pairs between the GA at the 5′ base of the stem (in the conserved A/GUGA sequence) and the GA at the 3′ base, and pyrimidine pairs flanking these two (Fig. 3 and Refs. 49 and 50). Similar nonstandard base-pairing features have been shown to serve as binding sites for several sequence- and structure-specific RNA-binding proteins. Finally, there can be two alternative arrangements in the hairpin loop, designated form 1 or form 2 (51–53). In form 1 SECIS elements, including those of D1 and GPX, the conserved adenosines are contained in a simple open loop. However, the D2 and D3 SECIS elements are predicted to form additional secondary structure in this region, with the adenosines located in a bulged region (Fig. 3, Form 2).

Additional mechanistic insights into SECIS function were gleaned from the D1-activity assay. These studies revealed that a SECIS element in the 3′ UTR could direct incorporation at any upstream in-frame UGA codon, and at multiple UGAs within an mRNA, provided a minimal spacing requirement was met (42). It was further shown that increasing the spacing between UGA and SECIS element by the insertion of 1.5 kb had no effect on SECIS activity. However, deletions that narrowed the spacing between UGA and SECIS to less than approximately 60 nucleotides (nt) abolished Sec incorporation (46). This may be due to steric constraints between the complex of factors assembled at the SECIS element (see *Section II.B*) and the ribosome decoding the UGA codon. At the other extreme, the identification of the human D2 mRNA with a UGA to SECIS spacing of nearly 5 kb indicates the upper limits for this distance may be very large (18).

B. Trans-acting factors are recruited by the Sec insertion sequence (SECIS) element to catalyze Sec incorporation

Studies of Sec incorporation in prokaryotes lent considerable insight into investigation of this process in eukaryotes. Homologs of the selC (tRNA[Ser]Sec) gene were identified in many species (54). Homologs of selD have also been identified in several eukaryotic species, some of which contain two selD genes, one encoding a selenoenzyme (Table 1). This provides a Se-dependent autoregulatory step in Sec biosynthesis. Recently, candidate selA homologs have been identified and are currently under investigation. Finally, factors conferring the two functions of prokaryotic selB have re-

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cently been identified and characterized. Identification of the eukaryotic SECIS element as the essential feature required for conferring UGA recoding led to the proposal of a model whereby this element would recruit a factor or factors conferring Sec incorporation, analogous to prokaryotic selB (42). This in turn led to the search for such factors, employing RNA-protein interaction methods using wild-type and nonfunctional mutant SECIS elements to establish specificity. Nonetheless, progress on this front was slow (55–57). In parallel, progress in genomics provided databases in which to search for sequence homologs of prokaryotic selB. This resulted in identification of candidates in archaea (58), and subsequently, in lower and higher eukaryotes. Finally, these two lines of research converged with the identification and cloning of two factors crucial to Sec incorporation in eukaryotes. First, a SECIS-specific binding protein, termed SECIS binding protein 2 (SBP2), was purified and cloned and shown to function in Sec incorporation (59, 60). This factor is limiting in reticulocyte lysates and in the cultured cell lines examined. Addition of the factor *in vitro* or its expression *in vivo* increases the efficiency of Sec incorporation (60). Next, the elongation factor candidates in archaea and in mammals were shown to exhibit specificity for selenocysteyl-tRNA (22, 61, 62). The designation EFsec was proposed to reflect this specificity. A final piece of information begins to bring the puzzle together was the demonstration that the elongation factor interacts with the SECIS-binding protein (22). Thus, the two functions contained within a single prokaryotic factor, selB, recruitment by the prokaryotic equivalent of a SECIS element and selenocysteyl-tRNA-specific elongation factor activity, are distributed between two separate but interacting eukaryotic proteins.

A model emerging from these results is depicted in Fig. 4. According to this scheme, the SECIS element recruits SBP2, an event that could theoretically occur in the nucleus as soon as this region is transcribed. The SECIS-SBP2 complex could then recruit the EFsec-tRNA complex and deliver it to the ribosome in the coding region. Because the SECIS element is located in the 3′ UTR in eukaryotes, not in the coding region as in prokaryotes, it obviates the need for dissociation and reassociation of the SECIS-SBP2 complex with each incorporation cycle. A scheme such as this could potentially allow

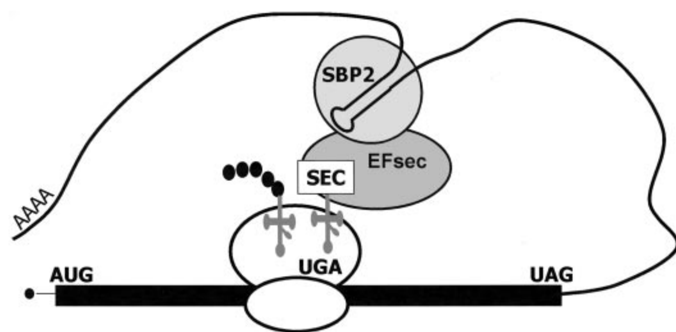

FIG. 4. Eukaryotic Sec incorporation directed from the 3′ UTR. The open reading frame of a eukaryotic selenoprotein mRNA is depicted by the *solid black bar*, with a ribosome decoding the UGA Sec codon. UTRs are indicated by the *thin black line*. The SECIS-SBP2-EFsec-tRNA complex is shown assembled in the 3′ UTR and looping back to the ribosome.

rapid reformation of SECIS-SBP2-EFsec-tRNA complexes from the two individual RNA-protein complexes after each EFsec-tRNA delivery cycle, *i.e.*, "reloading" for the next approaching ribosome. This would also be advantageous in the translation of a protein containing multiple Sec residues, such as selenoprotein P (63, 64).

Current and future studies in the field of selenoprotein synthesis will need to address the mechanics and kinetics of assembly of the UGA decoding complex, the *in vivo* efficiency of Sec incorporation, and how and to what extent termination is avoided at these codons. All of these steps are highly relevant for regulation of the activation and inactivation of the prohormone T_4.

III. Specific Biological Properties

A. Type 1 iodothyronine deiodinase (D1)

D1 was the first to be recognized by biochemical assays of T_4-to-T_3 conversion and was also the first to be cloned. Accordingly, a good bit more is known about its biochemistry than that of D2 and D3. D1-catalyzed T_4-to-T_3 conversion supplies a significant fraction of the T_3 in plasma of euthyroid humans and even more in the thyrotoxic patient (see *Section V.B*). A critically important characteristic of D1-catalyzed deiodination is its sensitivity to inhibition by PTU (6). This made initial demonstrations of the specificity of the T_4-to-T_3 conversion reaction easy to confirm (65–67). In addition, it allowed an explanation for the long-puzzling observation that thiouracil, the parent compound, partially blocked the effects of T_4, but not T_3, in experimental animals (6). Lastly, D1 is the only selenodeiodinase that can function as either an outer (5′) or inner (5) ring iodothyronine deiodinase, with D2 and D3 being (for all practical purposes) exclusively outer (D2) or inner ring (D3) deiodinases (Fig. 1 and Ref. 68).

1. Dio1 gene structure, chromosomal localization, mRNA and protein characteristics, and tissue distribution.

*a. Gene structure and chromosomal localization.*The elucidation of the *Dio1* gene structure was derived from studies comparing a polymorphism in the *Dio1* gene between the C57/BL6J and C3H/HeJ mouse strains (69, 70). The human gene is found on chromosome 1 p32–p33, in a region syntenic with mouse chromosome 4, the location of mouse *Dio1* (71). The mouse and human *Dio1* genes consist of four exons. The transcription start site is approximately 25 nt upstream of the initiator methionine. The UGA (Sec) codon is in exon 2, and the UAG (STOP) codon and the SECIS element are in the 953-nt fourth exon. The coding sequences of the mouse and rat D1 proteins are virtually identical. Both contain a Sec residue at position 126 (70).

b. D1 mRNA and protein characteristics. The complete cDNA sequences have been determined for rat, human, mouse, dog, chicken and tilapia D1 proteins (11, 70, 72–75). The mRNA sizes are about 2–2.1 kb and all contain a UGA codon in the region encoding the active center, which is highly conserved among species (Fig. 2). The cDNA encodes a protein of about 27 kDa that is highly similar in size (26–30 kDa) and sequence with a few informative exceptions (76). Depending on the

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detergent used, the molecular mass of the solubilized wild-type enzymes is about 50–60 kDa, suggesting that it may be a homodimer, although it is not yet certain that homodimerization is required for its catalytic activity (see *Section III.A.3* and Refs. 77–80).

c. Tissue distribution. By Northern analysis, D1 is expressed in many tissues of most vertebrates but not in amphibia (27, 81, 82). In the rat, these include liver, kidney, central nervous system (CNS), pituitary, thyroid gland, intestine, and placenta. In humans, D1 activity is notably absent from the CNS but is present in liver, kidney, thyroid, and pituitary and mRNA in circulating mononuclear cells by RT-PCR (83, 84).

2. Subcellular localization and topology. The D1 monomer is a type 1 integral membrane protein oriented with a 12-amino acid NH_2-terminal extension in the endoplasmic reticulum (ER) lumen and a single transmembrane domain exiting the ER at about position 36 (Fig. 5 and Ref. 85). The hydrophobic nature of the NH_2 terminus suggests that this portion of the molecule is an uncleaved signal recognition sequence and incorporates both signal and STOP-transfer functions. The transmembrane domains of other proteins, such as 17α-hydroxylase (P450-17) or D3, cannot substitute for the NH_2 terminus of D1 even though these permit synthesis of a membrane protein. This orientation is in agreement with earlier studies showing that gentle trypsinization of kidney microsomes caused both loss of enzyme activity and *N*-bromoacetyl (BrAc)T_3 labeling (86–88). Studies of the *in vitro*-translated Sec126Cys mutant of rat D1 show that, although the NH_2-terminal and transmembrane portions of the enzyme are not catalytically active, their sequence is critical because even minimal exchanges of amino acids in the transmembrane domain reduce the efficiency of its transient expression. These mutations do not affect the catalytic function of the protein that is successfully synthesized (85).

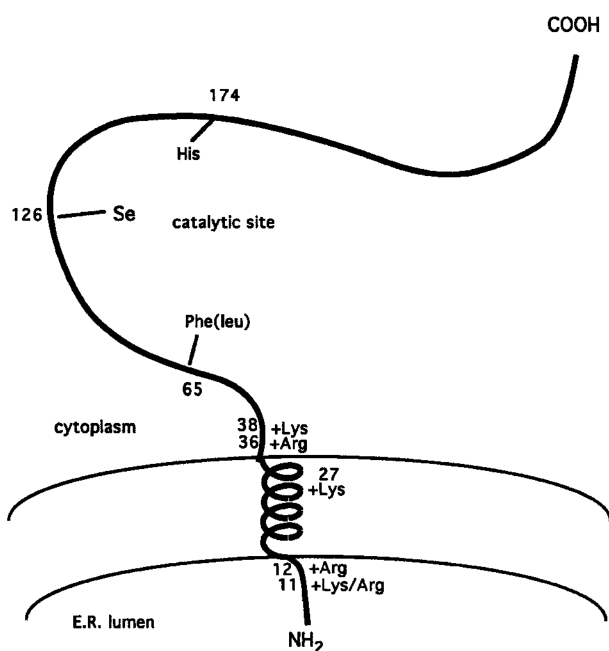

FIG. 5. The topology of the rat D1 as determined by protease sensitivities of the *in vitro*-translated rat Sec126Cys D1 mutant in the presence of pancreatic microsomes. Shown are locations of Phe65, important for rT_3, but not T_4, interaction with the active center, Sec126 and His174, which may be involved in maintaining Sec in a reduced state. [Reprinted with permission from N. Toyoda *et al.*: *J Biol Chem* 270:12310–12318, 1995 (85).]

The subcellular location of mature D1 is likely to be the plasma membrane, although this is still under study. This has been specifically demonstrated in the LLCPK1 proximal renal tubule cell line by $BrAcT_3$ labeling and enzyme markers and in pig thyroid cells by immunohistochemical studies using a primary D1 antibody (87, 89, 90). More recently, a basolateral plasma membrane location was confirmed in glial cells constitutively expressing a green fluorescent protein-tagged D1 (80). However, early studies of rat hepatocytes were conflicting, with some evidence suggesting that D1 colocalized with ER proteins such as protein-disulfide isomerase, nicotinamide adenine dinucleotide phosphate (reduced) cytochrome *c* reductase, and glucose 6-phosphatase and other results supporting a plasma membrane localization (88, 91). Recently, using either NH_2-terminal or COOH-terminal FLAG epitope-tagged, transiently expressed rat D1, confocal laser microscopy of transiently expressed D1 in the human embryonic kidney cell line (HEK293) or a mouse neuroblastoma cell line (NB2A) shows it located at the plasma membrane. It does not colocalize with the ER resident protein binding protein (BiP) as does D2 (Fig. 6 and Ref. 92). Furthermore, when either COOH- or NH_2-terminal FLAG-tagged D1 is transiently expressed in HEK293 cells that are then subjected to limited permeabilization of the plasma membrane with digitonin, the FLAG tag is visualized at the plasma membrane, even though BiP cannot be visualized. This observation confirms the earlier assignment of D1 to plasma membrane of kidney and thyroid cells. A preliminary report using transiently expressed green fluorescent protein-tagged D1 and D2 predicted an ER location for both enzymes (93). The reason for the discrepancies with the above-mentioned results with respect to the subcellular location of D1 is not clear (92). The location of D1 in primary hepatocytes remains to be determined.

Thus, because the topological studies predict that the catalytic site of D1 is cytosolic, a plasma membrane location could be viewed teleologically as offering ready access of circulating rT_3 and T_4 to the enzyme as well as facilitating the entry of the T_3 produced from T_4 into the plasma. The localization of D1 in the plasma membrane is in striking contrast to the ER localization of D2 in the same cell types using the same procedures (Fig. 6 and Ref. 92; see below). This differential subcellular localization of D1 and D2 may explain why there is such a minimal contribution of the T_3 generated by D1 to the intranuclear T_3 in contrast to the large fraction of D2-generated T_3 to this compartment (94, 95).

Early studies of rat kidney or liver D1 suggested the possibility that it was dimerized with a second protein, giving it a molecular mass of approximately 54–55 kDa (78, 96). It was not clear whether the enzyme was present as a homodimer or was bound to a protein of similar size. As mentioned, transient expression of a D1 enzyme in which the transmembrane domain has been deleted does not permit synthesis of functional protein (85). However, recent studies indicate that synthesis of a functional D1 enzyme lacking the

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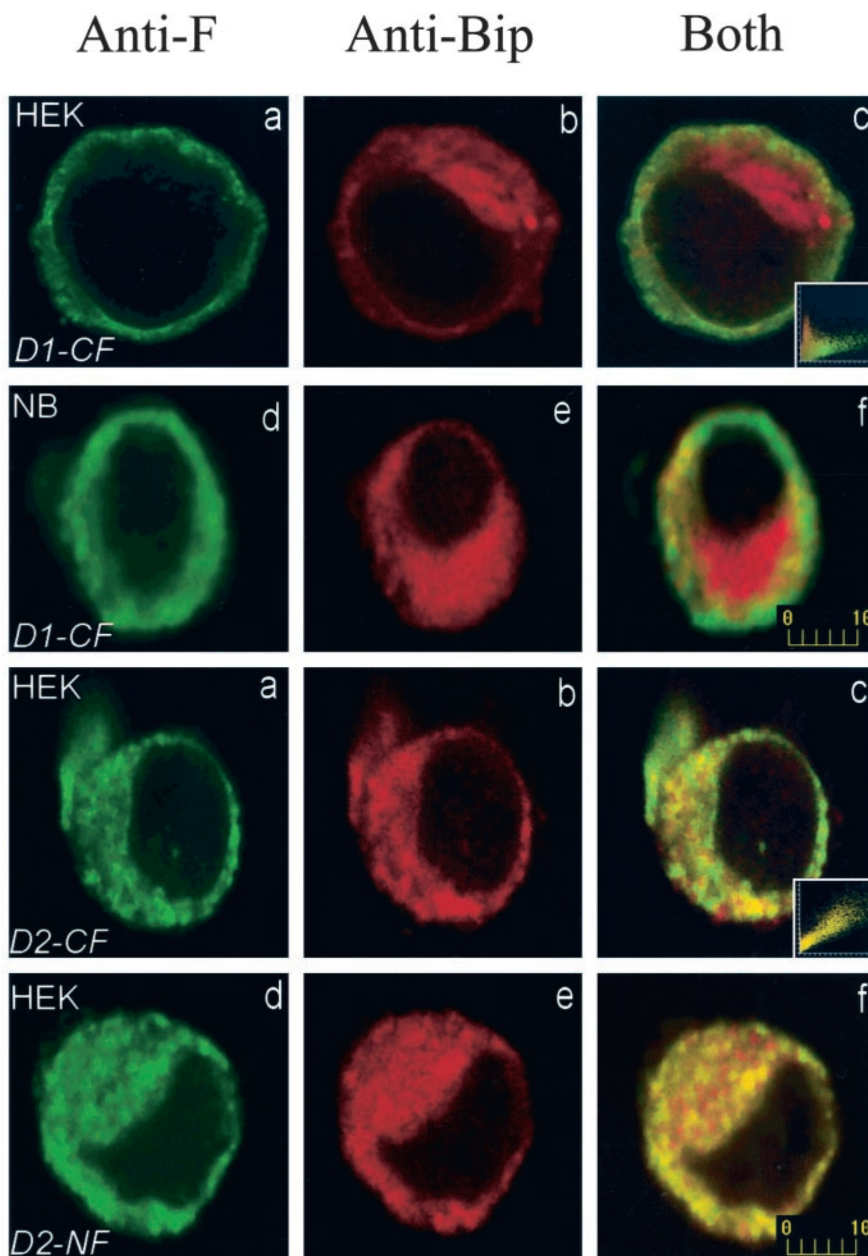

FIG. 6. Confocal microscopy of acetone-treated HEK293 or neuroblastoma cells transiently expressing Sec126Cys D1 fused with the FLAG peptide at the COOH terminus (*D1-CF*) or Sec133Cys D2 with a similar epitope. After fixation and acetone treatment, cells were incubated with mouse-anti FLAG and mouse anti-fluorescein isothiocyanate antibodies and costained with goat anti-GRP78/BiP and anti-goat-Rhodamine antibodies. Panels a–c and d–f are FLAG (*green*), GRP78/BiP (*red*), and superimposition immunofluorescence images of the same fields, respectively. The *inset* is the distribution spectrum of image pixels. Cell types are indicated in the *upper left corner* and transfected plasmid in the *lower left corner*. *Bar*, 10 μm. [Reprinted with permission from M. M. Baqui *et al.*: *Endocrinology* 141:4309–4312, 2000 (92). © The Endocrine Society.]

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NH_2-terminal transmembrane domain can occur if an intact, but catalytically inactive, D1 protein is also expressed in the cell (80). This suggests that homodimerization can occur between the cytosolic portions of D1 and that it is only necessary for one member of the homodimer to have a membrane anchor to permit the successful synthesis of functional D1. The amounts of intact D1 protein used to trap active, NH_2-terminal-deleted D1 were about 10-fold in excess of the quantity of the truncated protein. Accordingly, it is not certain whether homodimerization is required for enzyme function or whether dimerization and successful synthesis of a monomer without a transmembrane domain can occur when D1 is produced in large amounts.

3. Enzymatic properties and structure-activity relationships. Studies with both endogenous and recombinant enzymes indicate that the deiodination reaction catalyzed by D1 follows ping-pong kinetics with two substrates, the first being the iodothyronine, and the second being an endogenous intracellular thiol cofactor (30, 65, 66, 97–99). The first half-reaction deiodinates the iodothyronine leading to the formation of a putative selenoleyl iodide intermediate (Fig. 7). This is then reduced by an as yet unidentified intracellular thiol cofactor regenerating the enzyme. As indicated, PTU inhibits D1-catalyzed deiodination by competing with the putative thiol cosubstrate to form an essentially irreversible Enzyme-Se-S-PTU dead-end complex.

The important role of the nucleophilic properties of Se *vs.* S are illustrated by the roughly 100-fold lower turnover number for the Cys126 mutant of D1 relative to the Sec wild type (Table 2 and Ref. 30). This is similar to effects of this sub-

stitution in other selenoenzymes, such as formate dehydrogenase in bacteria (100). It should be noted, however, that the efficiency of the translation of the Cys126 is 50–100 times higher than SecD1 due to the inefficiency of selenoprotein translation in eukaryotes (see *Section II* and Ref. 30). Interestingly, a recent paper illustrates that deiodination of T_4 to T_3 with ping-pong kinetics can occur with a synthetic D1 enzyme. This protein was generated from a mouse monoclonal anti-T_4 antibody in which the OH groups of the four seryl residues of the variable region of the light chain were chemically replaced by Se, thus forming four Sec residues (101). The reaction was inhibited by PTU, which competed with dithiothreitol (DTT) just as in D1. This remarkable result serves to emphasize the importance of Sec in the deiodination reaction.

An important characteristic of D1 is its sensitivity to the potent nucleophilic reagent iodoacetic acid, which carboxymethylates the active center causing irreversible inactivation (Fig. 7 and Refs. 98 and 102). That this reaction is specific for the active-center Sec residue is shown by the protection of D1 from inactivation by iodothyronine substrates. Alternatively, a similar alkylation-based inactivation occurs using iodothyronine derivatives containing $BrAcT_3$, rT_3, or T_4 (Fig. 8). If these iodothyronines are labeled with ^{125}I, the protein will be covalently labeled (103, 104). The BrAc-iodothyronine derivatives also specifically label other iodothyronine binding proteins (*e.g.*, T_4-binding globulin, transthyretin, and TR), but also nonspecifically "alkylate" nonthyroid hormone binding proteins in microsomal preparations of rat liver such as protein disulfide isomerase (104). The specificity of the D1 labeling is established by the fact that D1 substrates block labeling of the 27-kDa D1 monomer but not that of other proteins (Fig. 8 and Refs. 25, 89, and 105). The blockade of this labeling by substrates or competitive inhibitors, such as gold thioglucose (GTG), confirms that $BrAcT_3$ or T_4 interacts with the substrate binding site. Gold is a competitive inhibitor (with iodothyronine) of the deiodination reaction, presumably interacting with the Se in the active center (11, 30). PTU does not block D1 labeling with $BrAcT_4$ unless D1 is first exposed to substrate because the first half-reaction is required for formation of the Enzyme-Se-PTU complex (106). The Sec126Cys mutant D1 can also be labeled by $BrAcT_4$, although the protection afforded by GTG is considerably less potent in agreement with the approximately 100-fold decrease in sensitivity of the Cys D1 mutant-catalyzed deiodination reaction to GTG (107). In fact, the loss of sensitivity to GTG of the Sec126Cys mutant and the relative insensitivity of D2 to inhibition by GTG led to the speculation that D2 would not prove to be a selenoprotein (108). This was based on the assumption that GTG would be as effective an inhibitor of any selenoenzyme as it was of GPX and D1 (11, 109). In fact, this assumption was not valid because both endogenous and recombinant D2 are 100-fold less sensitive to GTG than is D1. Furthermore, the kinetics are noncompetitive, implying that this inhibition is not due to interaction of GTG with the active center (110).

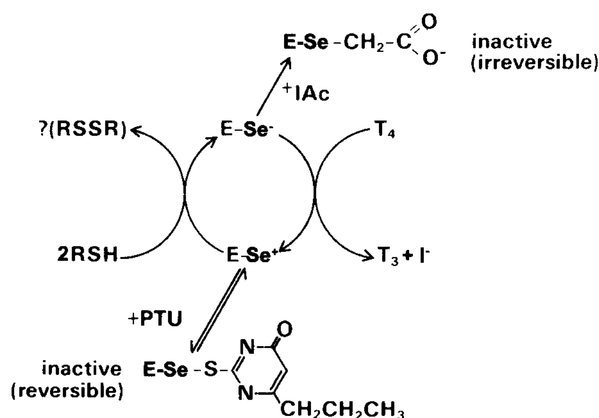

FIG. 7. Deiodination mechanism for D1-catalyzed T_4-to-T_3 conversion. The steps in the enzymatic reaction cycle at which iodoacetic acid and PTU are thought to act to inhibit catalysis are indicated. [Derived from Ref. 65.]

TABLE 2. Comparison of the translation efficiency and K_{CAT} of transiently expressed wild-type and Sec126Cys mutant D1 enzymes transiently expressed in COS-7 cells (108)

	Transiently expressed protein (fmol D1/mg cell protein)	K_{CAT} (min^{-1})
Wild-type rat D1	38	3300
Sec126Cys rat D1	690	31
Wild-type/Cys mutant	0.055	110

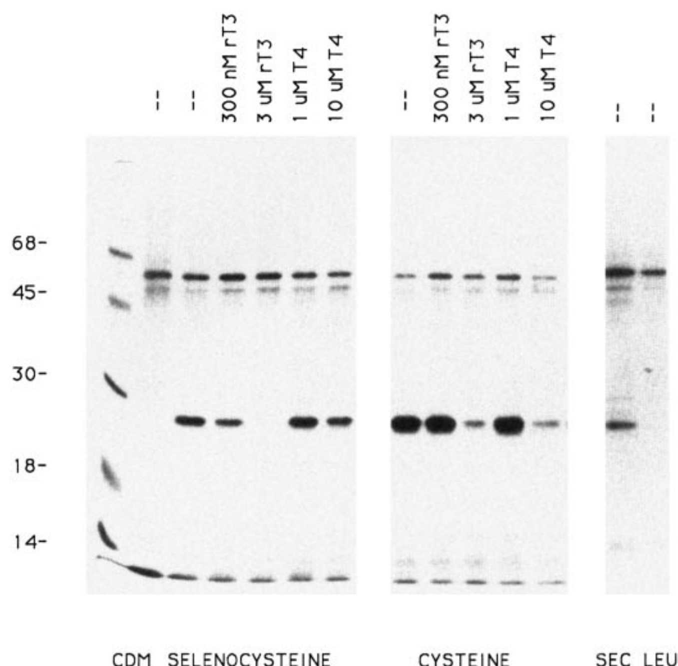

FIG. 8. Autoradiograph of a PAGE showing BrAc ^{125}I-T_4 labeling of transiently expressed wild-type, Sec126Cys, or Sec126Leu rat D1. The specificity of the 29-kDa D1 protein labeling is shown by the concentration-dependent reduction in signal when substrate is included in the reaction. The approximately 56-kDa band [probably protein disulfide isomerase (95)] is present in cells transfected with empty vector (CDM), and the Leu mutant and its labeling is not affected rT_3 or T_4. The expression of the wild-type (Sec-containing) protein is significantly lower than that of the Sec126Cys mutant as reflected in the density of the CysD1 bands. A Sec126Leu D1 mutant (*far right lane*) does not interact with $BrAcT_4$, indicating a Sec or Cys in the active center is required for covalent binding. [Reprinted with permission from P. R. Larsen and M. J. Berry: *Thyroid* 4:357–362, 1994 (510).]

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There is high conservation of the amino acids in the active center of D1 in various species (Fig. 2). The only exception to this is in the tilapia D1, in which proline replaces serine at position 128 (75). Because this is also characteristic of the PTU-insensitive D2 and D3 enzymes (Fig. 2), site-directed mutagenesis was used to replace this proline with serine (75). However, PTU sensitivity was not restored by this substitution, indicating that the explanation for the PTU insensitivity of tilapia D1 lies elsewhere in the protein sequence.

Kinetic studies have recently been performed using a rat D1 enzyme in which the vicinal Cys at position 124 of the active center was replaced by the alanine (Ala) found in D2 (Fig. 2) to test whether this residue is involved in catalysis by D1 (111, 112). The rat Cys124Ala D1 protein had a 10- to 15-fold higher apparent Michaelis-Menten constant (K_m) for DTT than wild type, suggesting that the SH group of this Cys residue was involved in the interaction with the second substrate. However, the maximum velocity (V_{max}) and K_m of the C124A mutant was not significantly different, although there was a 2-fold increase in the K_i for PTU. This supported a reaction mechanism for the D1 enzyme in which DTT interacts with the vicinal Cys to facilitate reduction of the oxidized Se in the active center (111). However, this mechanism, as is the case for that shown in Fig. 7, must remain speculative due to the lack of structural information.

In addition, Cys194 in D1 is conserved in all three deiodinase classes, suggesting an important role for this residue (Fig. 9). Replacement of this residue in D1 with Ala caused a modest increase in the K_m and decrease in V_{max} for rT_3 (112). Interestingly, neither the Cys124Ala nor the Cys194Ala mutations affected the rate of deiodination in cells transiently expressing these mutant D1 enzymes, suggesting that the increase in the K_m of the Cys124 mutant for DTT and the decreased V_{max} observed *in vitro* are not rate limiting *in vivo* (112). This could occur if reactivation of D1 by an endogenous thiol cofactor is very slow or does not occur *in vivo* (see below).

D1 catalyzes the deiodination of both the outer and inner ring of T_4 equally effectively, and this is influenced by pH (reviewed in Ref. 77). Interestingly, conjugation of the phenolic hydroxyl with sulfate markedly enhances the suitability of the iodothyronine substrates for D1-catalyzed 5 deiodination (113, 114). This is reflected in a markedly higher V_{max}/K_m ratio for those substrates. For example, with respect to T_4, the V_{max}/K_m ratios for ORD (13 μM·min/pmol·mg protein) or IRD (9 μM·min/pmol·mg protein) are similar, suggesting that these reactions occur at equal rates. The V_{max}/K_m ratio for 5 deiodination of T_4 sulfate is 2020 μM·min/pmol·mg protein (115). Sulfation of T_3 also markedly enhances its IRD, but the preference of D1 for T_3 sulfate

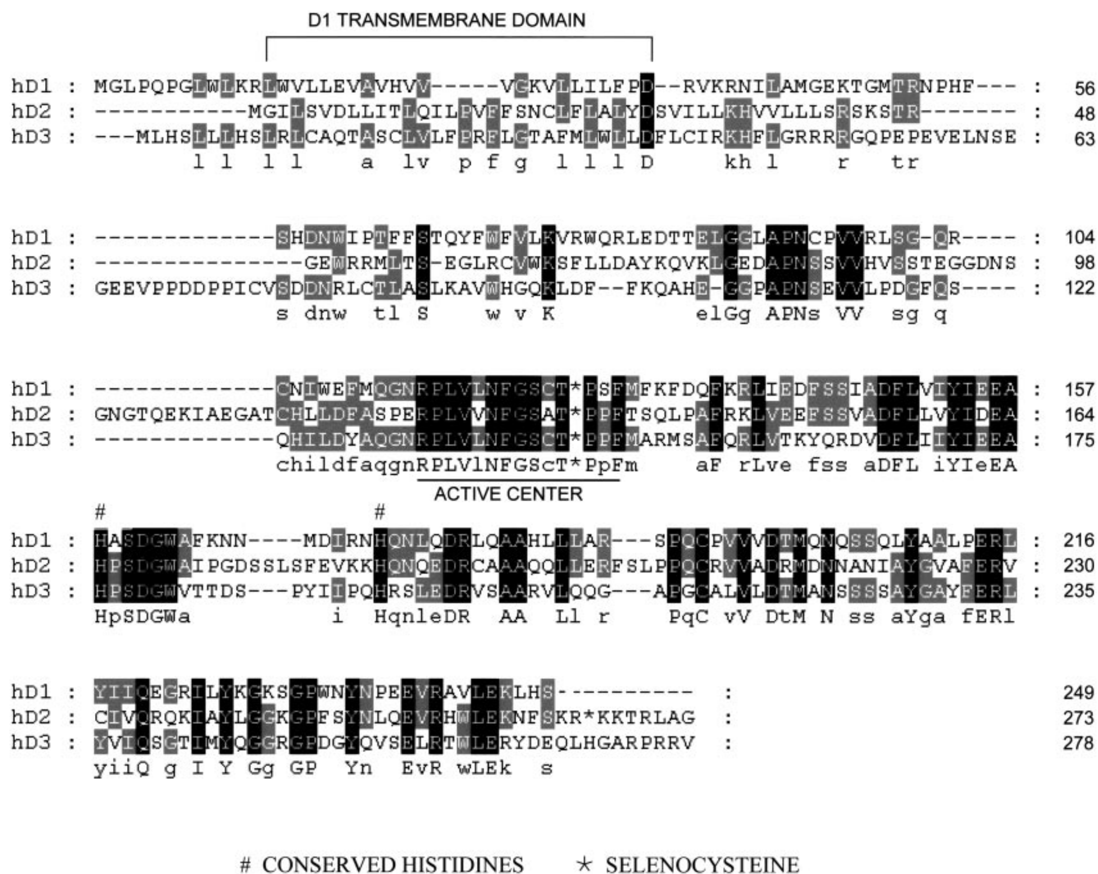

FIG. 9. Comparison of the deduced amino acid sequences of the three human iodothyronine selenodeiodinases. This arrangement illustrates the similarity of several specific regions of the three enzymes and is representative of the deiodinases in all species. The transmembrane domain of D1 is *overlined*, and *asterisks* indicate Sec residues. Note the second Sec residue in D2, 8 amino acids from the COOH terminus. Note also the conserved His residues corresponding to positions 158 and 174 in human D1.

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(T_3S) is much lower than is that for rT_3 or rT_3S. These analyses indicate that sulfation is a critically important modification of T_3 and T_4 because it facilitates rapid inactivation by IRD. Thus, the rate-limiting steps in the sulfation or desulfation of iodothyronines in a given tissue also need to be kept in mind when trying to predict the effects of D1-catalyzed iodothyronine deiodination (115).

Comparisons of the D1 enzymes of different species have led to the recognition of other structurally important amino acids. For example, the phenylalanine at position 65 is critically important for 5′ deiodination of rT_3 and 3,3′-diiodothyronine sulfate (T_2S) but not for deiodination of substrates with two iodines on the inner ring (73, 116). This was demonstrated by selective mutagenesis of the dog D1, which has an approximately 30-fold higher K_m for rT_3 than human or rat enzymes and contains a leucine at this position. It suggests a specific interaction of the inner ring of rT_3 and 3,3′-T_2S with Phe65, possibly through π-π interactions of the two aromatic rings, which is permitted by the absence of the bulky I atom at position 5.

More detailed studies of the effects of various differences in amino acid sequences between the human and dog D1 enzymes on substrate specificity have led to further insights into structural-activity correlations. Despite the roughly 20-fold higher V_{max}/K_m ratio for 5′ deiodination of rT_3 by human than dog D1, the V_{max}/K_m ratios for IRD of T_3S and 3,3′-T_2S were comparable for the two D1 enzymes (116). This indicated the major decrease in catalytic activity toward rT_3, due primarily to the Phe65Leu substitution in canine D1, does not affect IRD of these sulfated substrates. Although the reinsertion of the missing TGMTR peptide (residues 48–52 of human or rat D1) into dog D1 does not enhance rT_3 deiodination, it causes a marked decrease in the ORD of T_2S. This could be due to interference with the interaction of the SO_4 group with the active center. Nonetheless, taken together, these results lead to the surprising conclusion that these five residues are not critical to D1 function.

There are four histidine (His) residues in rat D1. Early studies showed that modification of one or more of these by diethylpyrocarbonate or rose bengal caused marked inhibition of deiodination (117). Systematic site-directed mutagenesis of these residues showed that His158 is critical for normal enzyme structure, whereas mutagenesis of His174 to glutamine or asparagine causes a 20- to 100-fold increase in the K_m for rT_3 (118). Subsequent comparisons of the dog, mouse, and human D1 proteins with the rat D1 shows that only these two His residues (158 and 174) are conserved in all four species (11, 70, 73).

The necessity for these indirect approaches to structure-activity correlations reflects the fact that the selenodeiodinase enzymes are integral membrane proteins, and thus, their crystallization in an active form is quite challenging. It is possible, for example, that the role of His174 is to maintain the reducing environment for the Se active center, a conclusion that is not apparent from inspection of its linear sequence.

4. Regulation of D1 synthesis.

a. Thyroid hormone. There are a number of substances, agents, or conditions that can influence the rate of D1 synthesis, the most potent being thyroid hormone (Table 3 and Refs. 11, 70, and 119–122). Thyroid hormone-induced increases in D1 activity and/or mRNA are well documented in rats, mice, and humans (11, 121). This is due to increased transcription, which in the human *Dio1* gene can be attributed to the presence of two thyroid hormone response elements (TREs) in the 5′-flanking region (FR) of the gene (Fig. 10 and Refs. 123–125). One of these, TRE-2, is a typical direct repeat with 4 bp separating the RXR-T_3 TR binding half-sites (DR+4). It is formed due to a polymorphism in an Alu

TABLE 3. Physiological influences on D1 activity

Conditions	Effect	Mechanism
Hormones and Second Messengers		
Thyroid hormone	Increase	Transcription
RA	Increase	Transcription
Glucocorticoid (*in vivo*)	Decrease (?)	?
(*in vitro*)	Increase	Transcription
cAMP-thyroid	Increase	Transcription
TSH-thyroid	Increase	Transcription
IL-1β, interferon γ	Decrease	Transcription
TNFα	Decrease (?)	Transcription
Nutritional		
Selenium deficiency		
Rat liver, kidney	Decrease	Translation
Rat brain, thyroid	No change	Resistance to Se depletion
Human (dietary)	Decrease	Translation (?)
Caloric intake		
Fasting (young rat)	Decreased D1 mRNA	Central hypothyroidism
Fasting (obese rat)	No change	Increased fat; protein spared
Diabetes (rat)	Decrease	Transcription Cofactor depletion (?)
Development		
Fetal tissues except rat intestine	Decrease	Transcription
Species Differences		
C3H/HeJ-BALB/cByJ mice	Decrease	Transcription
Human cerebral cortex	Absent	Transcription
Amphibians	Absent	Genetic (?)

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FIG. 10. Sequence of the promoter and 5′-FR of human *Dio1*. The *shaded area* indicates an Alu sequence. TRE-1 and TRE-2 and the two SP-1 sites are also indicated. [Reprinted with permission from C. Zhang *et al.*: *Endocrinology* 139:1156–1163, 1998 (125). © The Endocrine Society.]

sequence and is present 660 nt 5′ to the transcription start site [TSS (125)]. TRE-1 is an unusual element in which two TR-binding octameric half-sites are separated by 10 bp. Both of the octamers binding TR have a pyrimidine at their most 5′ position, this being the highest affinity TR-binding DNA half-site (123). Both TREs contribute to the response of the human *Dio1* promoter, and methylation interference binding studies show that the unconventional TRE-1 binds TR but not RXR (123). Studies in TR-knockout mice indicate that TRβ is primarily responsible for T_3-mediated D1 stimulation (126). Given these results, one would expect that the T_3 responsiveness of the human *Dio1* gene would be most obvious in patients with thyrotoxicosis, such as in Graves' disease. In fact, semiquantitative PCR of D1 mRNA in human peripheral blood mononuclear cells demonstrates it is increased in proportion to the degree of hyperthyroidism (84). As discussed below, this can explain the marked increase in PTU-sensitive plasma T_3 production in patients with hyperthyroidism (127).

Although both the rat and mouse liver D1 mRNAs are markedly increased by T_3, canonical TREs have not yet been identified in the available 5′-FR of these genes (our unpublished data and Refs. 70 and 128). The response of the *Dio1* gene to T_3 in FRTL5 cells and in a rat pituitary cell line (GC) is due to transcriptional activation and is not blocked by cycloheximide, indicating that this is a direct effect of T_3 not requiring synthesis of an intermediate protein (122).

b. RA. RA increases the concentration of D1 in human thyroid carcinoma cell lines (129). This can be accounted for by the TREs in the human *Dio1* gene that also respond to RA (Fig. 10 and Refs. 124, 125, and 130).

c. Glucocorticoids. Acute administration of glucocorticoids to humans or rats decreases the ratio of circulating T_3 to T_4, implying that these agents block T_4-to-T_3 conversion (131, 132). Hepatic D1 in liver homogenates decreases in dexamethasone-treated rats, but in spheroid cultures of primary rat hepatocytes, glucocorticoids enhance the induction of the D1 mRNA induced by T_3. This occurs despite the fact that dexamethasone alone causes only a modest increase in D1 activity (133). The dexamethasone-induced increase in D1 mRNA was blocked by pretreatment of the cells with cycloheximide, indicating that ongoing protein synthesis is required for this effect. In rats, the fall in T_3 that follows the administration of dexamethasone may be explained by a decrease in the plasma T_3 production rate and in the fractional conversion of T_4 to T_3 (134, 135). However, more recent studies in humans indicate that D3 activity is induced by dexamethasone, and the acute decrease in serum T_3 that follows a high dose of glucocorticoids may be due to an increase in D3-mediated T_3 clearance via 5 deiodination (136).

d. Gonadal steroids. Although no direct studies of effects of gonadal steroids on D1 activity have been performed, D1 activities are higher in male than in female rat liver, and this difference is eliminated by gonadectomy (137, 138). However, there are no gender-related differences in D1 content in the kidney.

e. GH. Treatment of euthyroid adults with GH increases the ratio of plasma T_3 to T_4 and reduces that of rT_3 to T_4 (139). The mechanism for this is peripheral because it is found in T_4-replaced individuals with central hypothyroidism. It could be a consequence of enhanced D1 activity or due to a reduction in D3, analogous to the effect of GH to reduce D3 activity in chicken liver (140, 141).

f. cAMP. Studies in the FRTL5 rat thyroid cell line have shown a 3-fold increase in D1 mRNA induced by TSH, which is replicated by $(Bu)_2$cAMP or forskolin. The effects of these agonists were additive to that of T_3, the combination resulting in a 5-fold stimulation relative to control (142). This could not be explained by an alteration in D1 mRNA disappearance rate, and the effect was blocked by cycloheximide, indicating that persistent protein synthesis is required for the effect. The mechanism for the stimulation of rat *Dio1* transcription by cAMP has not been elucidated.

g. Cytokines. IL-1, IL-6, TNFα, and other cytokines have been postulated as potential mediators of the alterations in thyroid function that occur during severe illness (143–145). TNFα, IL-1β, and interferon γ decrease D1 activity and mRNA in FRTL5 cells, although TGFβ has no effect (142). The effects of TNFα have been examined in hepatocytes and HepG2 cells with contradictory results. TNFα decreased the T_3-stimulated D1 mRNA in HepG2 cells (146). This effect is blocked by dominant-negative nuclear factor κB (NF-κB) coexpression and also by inhibition of the TNFα-induced activation of NF-κB by clarithromycin, suggesting that it is related to the TNFα-induced increase in NF-κB. NF-κB impairs the function of a number of hormonal ligand-directed transcriptional stimulators, although no direct interaction

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between TR and NF-κB has been demonstrated. In a second study in dispersed rat hepatocytes, IL-1β and IL-6 blocked the T_3 induction of D1 mRNA and activity but TNFα had no effect (147). The T_3 effect with IL-1β was rescued by coexpression of the nuclear steroid receptor coactivator (SRC-1) but not by cAMP response element binding protein-binding protein or cAMP response element binding protein-binding protein-associated factor. Because IL-1 does not affect the amounts of SRC-1 in the hepatocytes, the effect was attributed to competition between IL-1 and T_3-stimulated transcriptional events for limiting quantities of SRC-1. This was supported by evidence that IL-1 and IL-6 reduced T_3 induction of Spot-14 and malic enzyme mRNA as well as D1, and that SRC-1 coexpression also rescued these as well as IL-1-suppressed, glucocorticoid-induced mouse mammary tumor virus promoter activity.

The differences in the effects of TNFα in these two studies could be due to differences in the experimental paradigms. Despite this, both studies suggest that one mechanism for an acute decrease in D1 expression during illness could be competition for limited amounts of one or more transcription factors that are rate limiting for both cytokine- and T_3-dependent transcriptional events. IL-1β stimulates D1 and D2 and TNFα stimulates D2 in rat pituitary cells and GH-3 cells (148). If this occurs in the thyrotrophs, it could act to reduce TSH synthesis and release in severe illness.

h. Se deficiency. The decrease in hepatic D1 activity in liver of Se-deficient rats and the demonstration that D1 could be labeled with ^{75}Se were the first clues that this trace element was critical to the function of this enzyme (12–14, 149). However, the effects of Se deficiency are complex due to a combination of factors. First, Se retention during dietary deficiency differs among different tissues high in brain, pituitary, thyroid, adrenals, and gonads. In contrast, dietary Se deficiency rapidly reduces the Se content of plasma, liver, skeletal muscle, and heart (150–152). Thus, the effect of Se deficiency on the synthesis of intracellular selenoproteins, such as the selenodeiodinases and GPX, will depend on the tissue being examined. For example, in rats with Se deficiency, thyroidal D1 activity is preserved, whereas that in the liver drops precipitously (152). In the intact rat, Se deficiency is generally associated with an increase in serum T_4 concentrations but little change in serum T_3 concentrations, effects that are analogous to the situation in the D1-deficient C3H mouse (153). Se deficiency also decreases D1 in kidney but this is accompanied by a decrease in D1 mRNA, which does not occur in the liver (154). Se deficiency is observed in patients receiving diets that are restricted in protein content, such as those given for phenylketonuria, and has also been found in elderly patients (155–158). In Se-deficient humans, the serum T_4 and T_4 to T_3 ratios are mildly elevated, but TSH is normal. In one endemic goiter region in Africa, there is an accompanying Se deficiency (159, 160). When Se was resupplied to these iodine-deficient individuals, there was a deterioration of thyroid function as evidenced by an increase in TSH and a reduction in serum T_3, suggesting that the reduction in D1 during Se deficiency can protect against iodine deficiency, presumably by reducing the IRD of T_4, T_3, or T_3S (161, 162).

There have been numerous studies of the effects of Se deficiency on thyroid status in rats, as researchers have attempted to determine the role of hepatic and renal D1 in plasma T_3 production. There is general agreement that hepatic D1 is markedly reduced by Se deficiency but that thyroid and pituitary D1 are not (12, 13, 150, 152). Unexpectedly, there is little change in serum T_3 despite 10–20% increases in serum T_4 in intact animals, and serum rT_3 and T_3S are generally increased (163–165). These results argue that hepatic and renal D1 make minimal contributions to plasma T_3, but the results are confounded by the roughly 40% contribution of thyroidal T_3 secretion to this pool in the rat (see *Section V.B*).

To eliminate this problem, the effects of Se deficiency have also been examined in thyroidectomized T_4-replaced rats. Such studies are analogous to those performed with PTU in which approximately 50% inhibition of extrathyroidal T_4-to-T_3 conversion is found (6, 166–168). The results of the Se-depletion studies are conflicting. In one, Se deficiency caused no decrease in plasma T_3 despite a greater than 93% decrease in hepatic D1 (163). This result led to the conclusion that the thyroid gland must be the major source of circulating T_3 in the rat. However, that conclusion did not take into account the contribution of D2-catalyzed T_4-to-T_3 conversion in tissues resistant to Se depletion to plasma T_3 (169). In a later study in T_4-replaced rats, an approximately 25% decrease in serum T_3 and 32% decrease in total T_3 production in Se-deficient rats was found, similar to the effects of PTU in the same study (170). The reasons for the more modest decreases in the serum T_3 during PTU treatment in this study than the 60% typically observed are not clear. The overall conclusion of these studies is that thyroidal T_3 secretion provides about 40% of the plasma T_3 in the rat and that approximately 50% of extrathyroidal T_4-to-T_3 conversion is catalyzed by D1. This is consistent with estimates of approximately equal contributions of D1 (PTU sensitive) and D2 (PTU resistant) to extrathyroidal T_3 production in rats generated by sophisticated kinetic techniques (169).

i. D1 expression is reduced in fetal tissues. It is well recognized that the serum T_3/T_4 ratio in the fetus and newborn is quite low relative to infants even a few hours older (171). This is likely due to the high hepatic D3 expression in the human fetus, together with placental D3 expression. Hepatic D3 expression disappears in late fetal life (172). The abrupt increase in T_3 levels that occurs in the first hours after delivery and the higher ratio of T_3 to T_4 that is maintained thereafter is probably, then, due to a combination of factors: a rapid increase in TSH inducing both T_3 and T_4 secretion from the thyroid, and the absence of the placenta. There may also be increases in D1 and D2 (173). Changes in deiodinase activity have been investigated in the developing rat, and in general, D1 activity is low in all tissues of the fetal rat. It begins to appear soon after birth in the intestine, liver, kidney, cerebrum, cerebellum, and gonads (174). D1 activity is higher in the skin of the newborn rat than in the 2-wk-old or adult rat, in which it is virtually undetectable. D1 is the major deiodinase activity in liver, kidney, and intestine at all stages of life in the rat, and these tissues presumably are the most active in the PTU-sensitive conversion of T_4 to T_3. Because the

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age-related differences are also apparent using RT-PCR measurements of rat D1 mRNA, they arise at a pretranslational level. The mechanism for the age-related effects on D1 expression is unknown. The physiological purpose served by the low D1 activity in the fetus is thought to be to reduce circulating T_3, thus permitting the changes in intracellular T_3 to be determined by the developmentally programmed changes in D2 and D3 activities (Ref. 173; see *Section V*).

j. Nutritional influences on D1 expression. A decrease in the concentration of circulating T_3 relative to that of T_4 and an increase in rT_3 concentrations in fasting humans was one of the earliest indications that the peripheral metabolism of thyroid hormones in humans could be modulated by physiological or pathophysiological events (175). Similar changes are apparent in the acutely ill patient (176, 177). Thyroidal secretion accounts for only about 20% of T_3 production in humans (2). Therefore, the acute reduction in serum T_3 during fasting or illness to less than 50% of its baseline concentration must derive, at least in part, from impaired T_4-to-T_3 conversion by D1 or D2 or by an increased T_3 clearance by D3. Analysis of the effects of illness and fasting are discussed further in *Section VI*, but data in studies with rodents are reviewed here.

Early studies of D1 activities in livers from fasted rats suggested that an impairment of T_4-to-T_3 conversion might be a consequence of a reduction in the thiol cofactor, which serves as the cosubstrate for D1 catalyzed T_4-to-T_3 conversion (120, 178). Despite three decades of research, this cofactor has not been identified. The rat has been used extensively as a model for the effects of fasting and illness on T_4-to-T_3 conversion in humans. Unfortunately, the young adult rat is a poor model for the effects of starvation in humans because of the low body fat content. Serum T_3 does fall rapidly in the 8-wk-old fasted rat but so also does serum T_4 (179). Surprisingly, despite reduced D1 in rat liver, the total body conversion of T_4 to T_3 in the rat is not reduced by fasting (180, 181). It is also well recognized that fasting is a severe stress to the 8-wk-old rat, which rapidly loses protein nitrogen during the first few days and succumbs after approximately 5 d (179). This is associated with a marked reduction in serum TSH, T_4, and T_3 concentrations, *i.e.*, central hypothyroidism probably in part due to leptin deficiency (182). In contrast, if 16-wk-old rats with larger fat stores are fasted, the nitrogen loss due to protein catabolism is delayed and serum T_4 falls less rapidly and to a lesser degree, allowing studies up to 10 d of starvation. Even more impressive is the effect of prefeeding rats with high-fat diets to induce obesity before starvation. Under these circumstances, urinary nitrogen loss is markedly reduced during the period of fasting, serum T_4 and T_3 concentrations fall less then 30% over the first 5 d, and serum T_3 concentrations actually increase somewhat between 10 and 20 d of starvation (179).

This pattern differs markedly from that observed in starved humans, in whom circulating T_3 concentrations decrease rapidly to about 50% of control and remain low for up to 3 wk of fasting (183). Thus, not only does the central hypothyroidism of the acutely fasted, nonobese rat not resemble the pathophysiology of the human, it appears that even when this is prevented by providing increased fat stores to this normally lean animal, the pattern of changes in serum T_3 and T_4 in the circulation do not match those seen in humans. One must conclude then that studies in the rat (and probably mouse) are not likely to shed much light on the acute pathophysiological changes in thyroid hormone deiodination in fasting humans.

The reduction in D1 activity in the liver of the fasted rat is in part due to a decrease in D1 protein at a pretranslational level (184, 185). This can be prevented by maintaining a euthyroid status in these animals and, thus, presumably reflects the effect of the stress-induced central hypothyroidism. Similarly, the reduced serum T_3 concentrations in the diabetic rat can also be explained on the basis of decreased D1 mRNA in both kidney and liver but, in this case, the effect is reversed by insulin administration (185).

5. Regulation of D1 inactivation/degradation. Studies with protein synthesis inhibitors have indicated that the half-life of D1 in intact or transiently transfected cells is greater than 12 h (186–188). The inactivation and subsequent degradation of D1 is enhanced by substrates such as iopanoic acid or rT_3 (186). Substrate-induced inactivation is blocked by PTU, indicating that deiodination is required for the effect (187). The substrate-induced inactivation process consists of two phases. The early phase can be reversed by incubation of microsomes with high DTT concentrations. If longer times, *e.g.*, 6 h, are allowed to pass between substrate exposure and incubation with DTT, recovery of D1 activity is much less complete, indicating that an irreversible change has occurred (187). Studies with transiently expressed D1 tagged with a FLAG epitope confirm the substrate-induced acceleration of D1 inactivation. There was no associated decrease in D1 protein, nor was there ubiquitination of D1 such as occurs with D2 (188). It is not certain whether the inactivated D1 can be reactivated *in vivo*. If it cannot, maintenance of D1 activity would require continued synthesis of D1.

B. Type 2 iodothyronine deiodinase (D2)

D2 is an obligate outer ring selenodeiodinase that catalyzes the conversion of T_4 to T_3 and rT_3 to 3,3′-T_2. D2 has a K_m for T_4 in the nanomolar range under *in vitro* conditions in the presence of 20 mM DTT. The K_m *in vivo* is similar, given results in HEK293 or COS cells transiently expressing human D2 (189). As the most recently cloned of the three deiodinases, our knowledge as to its properties and function is still accumulating rapidly. For example, D2 was known to be particularly important in the brain, producing more than 75% of the nuclear T_3 in the rat cerebral cortex (190). The presence of D2 activity in human skeletal muscle, unexpected from studies in rats, provides a plausible source for a significant amount of the extrathyroidally generated plasma T_3 (110). Earlier results suggesting an important posttranslational regulation by substrate have been explained by the demonstration that D2 undergoes selective proteolysis via the ubiquitin-proteasome pathway. This pathway is markedly accelerated by interaction with substrate (188, 191, 192). The identification of the mouse *Dio2* gene has led to the generation of the first deiodinase-knockout mouse, allowing potential new insights into the physiological role of D2 (24).

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1. Dio2 gene structure, chromosomal localization, mRNA and protein characteristics, and tissue distribution.

a. Gene structure and chromosomal localization. The *Dio2* gene is present as a single copy located on the long arm of the 14th human chromosome in position 14q24.3 (193, 194). It is about 15 kb in size, and the coding region is divided into two exons by an approximately 7.4-kb intron. The exon/intron junction is located in codon 75 and is found at an identical position in the human and mouse *Dio2* genes (20, 193, 195). For the human gene, there are three TSS, 707, 31, and 24 nt 5′ to the initiator ATG. The longest 5′ UTR of the human D2 mRNA contains an approximately 300-bp intron that can be alternatively spliced (Fig. 11 and Ref. 195). Other splicing variants have also been identified involving the coding region (196). The human, mouse, and rat *Dio2* 5′-FR have been isolated and functionally characterized. All contain a functional cAMP responsive element (CRE), but only human *Dio2* has thyroid transcription factor-1 (TTF-1) binding sites (Fig. 12 and Refs. 195, 197, and 198).

b. D2 mRNA and protein. The cloning of a complete D2 cDNA was challenging due to its huge size. An RT-PCR-based method using oligonucleotides designed for conserved D1 and D3 regions provided the first D2 fragment from *R. catesbeiana* (16). The fragment was extended by 5′ and 3′ rapid amplification of cDNA ends (RACE), and the identity of the mRNA was confirmed by expression in oocytes. A partial rat cDNA containing the coding region and portions of the 5′ and 3′ UTR was subsequently isolated, leading to the identification of the coding region of the human cDNA (17). Both the rat and the human proteins were expressed *in vitro* and showed classical D2 kinetics (17, 110). However, neither of these clones included a SECIS element. The D2 coding region has also been cloned from a teleost fish, *Fundulus heteroclitus* (199). An approximately 5.1-kb rat D2 fragment has also been reported, but this also lacks a SECIS element (200). Human, mouse and chicken D2 cDNAs containing intact 3′ UTR (5–7.5 kb) were successfully identified using GenBank searches and library screening combined with 3′ RACE PCR. These D2 cDNAs encode functional D2 proteins in *X. laevis* oocytes and/or by transient expression (18–20).

The rat and human D2 mRNA are approximately 7.5 kb, and the chicken D2 mRNA is approximately 6 kb (17–19, 110). The approximately 7.5-kb band of the rat D2 mRNA appears as a doublet in different brain regions but not in brown adipose tissue (BAT). The difference in size between the 2 bands is approximately 500-1000 bp (17, 110). The same phenomenon is present in human thyroid, brain, and heart (195, 201). A detailed analysis involving nuclease mapping, 5′ RACE, primer extension, and Northern blots indicates that the human D2 mRNA can exist as four different transcripts in thyroid, brain, and possibly in other tissues (195). The longest transcript is approximately 7.5 kb; it starts from the most 5′ TSS, 708 nt 5′+ to the ATG, and is the only transcript found in placenta. A shorter, minor, approximately 7.2-kb D2 species uses the same TSS but the approximately 300-bp intron is spliced out. Two shorter transcripts of approximately 6.8 kb, differing by only 7 nt, utilize 3′ TSSs located close to the translation initiation site (Fig. 11). It is not known whether the rat and mouse genes utilize the same two major TSSs, but this is likely to be so for D2 in rat brain (17, 110, 195). The deduced amino acid sequences of the chicken, mouse, rat, and human D2 enzymes contain two Sec residues. The first is in the active center of the enzyme, whereas the second is located close to the COOH terminus (Fig. 9). In fish and frog D2, only the Sec codon in the active center is present (16, 17, 19, 20, 110, 199). The D2 SECIS elements are form 2 structures (Fig. 3) located close to the 3′ end of D2 mRNA and separated from the UGA codon in the active center by approximately 5 kb of A/U rich sequences (18–20). This is the longest separation between a Sec encoding UGA and SECIS

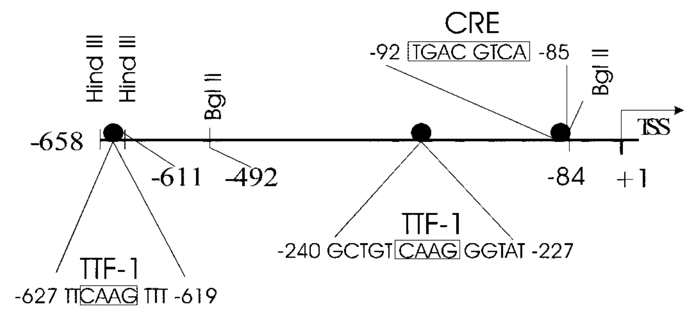

FIG. 12. Schematic diagram of the promoter and 5′-FR of the human *Dio2* gene. The CRE and functional TTF-1 binding sites are indicated. Only the 5′ TSS is shown.

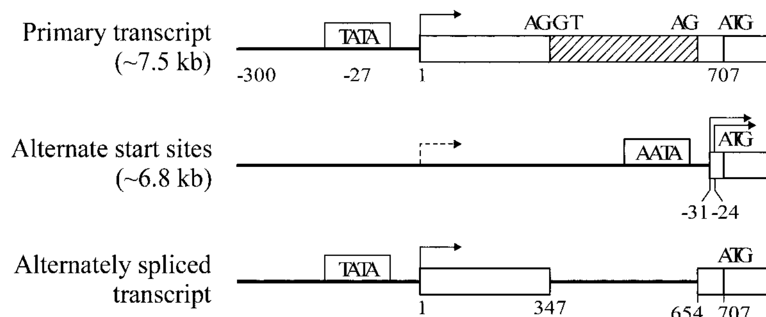

FIG. 11. Schematic diagram of the 5′ regions of the three predicted human D2 mRNA transcripts based on analysis by Northern blotting, primer extension, and S1 ribonuclease digestion. The position of the alternately spliced intron in the 5′ UTR is indicated. One proximal and two distal TSSs are used in human thyroid, cardiac muscle, and pituitary. In placenta only, the 5′ TSS is used, whereas in brain the intron may not be expressed, resulting in an mRNA of intermediate size. [Reprinted with permission from T. Bartha *et al.*: *Endocrinology* 141:229–237, 2000 (195). © The Endocrine Society.]

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element in any eukaryotic selenoprotein mRNA reported to date.

The deduced human D2 protein is approximately 31 kDa and contains a hydrophobic NH_2 terminus (Fig. 9 and Refs. 17, 19, and 110). The catalytic center is approximately 100% conserved between the frog, chicken, rat, and human enzymes (Fig. 2). The similarity of the chicken and human D2 proteins between the NH_2 terminus and the first Sec and between the first and second Sec residues is 88% and 90%, respectively (19). It is not known how often the COOH-terminal UGA codon is translated as Sec. The full-length cDNA is transiently expressed as a ^{75}Se-labeled doublet, but there are no kinetic differences between these two D2 proteins (202).

c. Tissue distribution. In the rat, D2 activity is predominantly expressed in the pituitary, brain, and BAT (9, 10, 203–206). D2 activity is also present in the rat gonads, pineal, thymus, mouse mammary gland, uterus of pregnant rat, and human coronary artery and aortic smooth muscle cells (174, 207–211). High levels of D2 mRNA and activity are found in the mouse cochlea at the eighth postnatal day, suggesting a role of D2 in generating T_3 for cochlear development (212). In cortex, D2 mRNA is predominantly expressed in astrocytes of the neonatal rat forebrain (213). D2 activity is also highly expressed in tanycytes, specialized ependymal cells lining the third ventricle with multiple D2 mRNA expressing cellular processes entering the median eminence regions (Refs. 213–216 and Fig. 13). A monosynaptic pathway has also been identified between the D2 expressing arcuate nucleus and the neuroendocrine TRH cells in the paraventricular nucleus (217).

Northern blots show that D2 expression in humans is more extensive than previously supposed. D2 mRNA and/or activity is expressed in the human thyroid, heart, brain, spinal cord, skeletal muscle, and placenta, and low-abundance D2 message is present in kidney and pancreas (17, 110, 195, 201, 218, 219). With the exception of thyroids from patients with Graves' disease and follicular adenomas, which may have particularly high levels of D2 mRNA and activity (201), the D2 mRNA levels are disproportionately high for the D2 activity usually found in the thyroid. This is likely due to the substrate-induced D2 proteolysis in the proteasomes (see *Section III.B.5*), which is probably amplified in the thyroid by T_4 secretion. Expressed sequence tag-derived D2 sequences are also present in libraries from prostate, breast, and uterus, although none of these have been reported to express D2 activity (18). D2 mRNA or activity are present in human pituitary and brain tumors (201, 220, 221), and D2 activity has been demonstrated in human keratinocytes (222) and mesothelioma cells (see below and Ref. 223).

The species-specific differences in D2 expression indicate that this enzyme has species-specific functions. D2 is the only 5′ deiodinase in the adult human CNS, unlike the situation in the rat (83). In contrast to human tissues, rat heart and skeletal muscles do not express D2 mRNA, and expression in the thyroid gland of the adult rat is very low (17, 198). D2 message is absent in human and rat liver (17, 110), but both D2 mRNA and activity are present in the adult chicken liver, and the D2 mRNA is found in the liver of teleost fishes *Fundulus heteroclitus* and *Oreochromis niloticus* but not in tadpoles (16, 19, 199, 224).

d. An alternative D2 candidate, p29 (p27). The specific labeling of D1 by BrAc derivatives of labeled iodothyronines has been discussed (Fig. 8). In the early attempts to identify D2 protein, cAMP-stimulated glial cells with increased D2 activity were exposed to $BrAcT_4$. A protein of 27–29 kDa (p29) was labeled, and this labeling was partially blocked by overnight exposure to 1 nM free T_4, although not by iopanoic acid (225). This result, together with indirect circumstantial evidence that D2 was not a selenoprotein, led to the hypothesis that p29, which

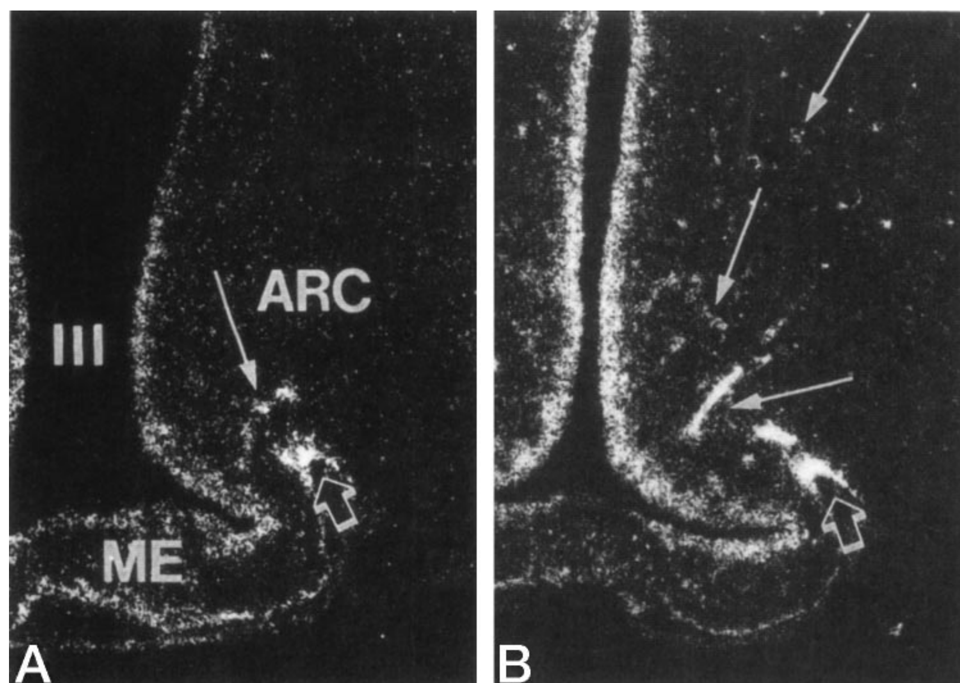

FIG. 13. High-power photomicrograph of D2 mRNA hybridization at the midlevel of the arcuate nucleus (ARC) in euthyroid (A) and hypothyroid (B) rats (dark-field illumination). Note intense hybridization in the ependymal cells in floor and walls of third ventricle (III) and in surrounding blood vessels in ARC, particularly in the hypothyroid animal, and at the base of hypothalamus over the tuberoinfundibular sulci (*arrowheads*). *Long arrows* denote hybridization associated with blood vessels in ARC. Original magnification, ×100. [Reprinted with permission from H. M. Tu *et al.*: *Endocrinology* 138: 3359–3368, 1997 (215). © The Endocrine Society.]

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has no catalytic activity, could be part of a larger multiprotein D2-containing complex, possibly serving as the substrate binding protein (226–228). Three major arguments support this position: 1) the failure of Se deficiency to reduce D2 catalytic activity either *in vivo* or *in vitro* (164, 229, 230), 2) the inability to identify a ^{75}Se-labeled protein of the expected size in cells expressing D2 (231), and 3) the inability to identify immunoreactive protein using antibodies prepared against peptides deduced from the sequence of the D2 mRNA (231). The conclusions of a number of published studies require the assumption of identity between D2 and the BrAc-iodothyronine-labeled p29 (232–234).

Despite these arguments, the evidence supporting the conclusion that the *Dio2* gene encodes the D2 enzyme is compelling and includes the following: 1) the protein transiently expressed in cells transfected with the human, rat, or chicken D2 cDNAs produces a approximately 31-kDa enzyme with the identical kinetic profile to endogenous D2; 2) the active centers of the six different D2 species cloned to date are virtually identical and highly similar to those of D1 and D3 (Fig. 2); 3) Northern blots show D2 mRNA in every tissue in which D2 activity has been found (17–19, 209); 4) in a number of different tissues, changes in D2 mRNA expression parallel those in D2 activity during circadian rhythmicity, stimulation by β-adrenergic agents, or in hypothyroidism, *i.e.*, pineal gland, brain, and skeletal muscle (209, 215, 235). In addition, D2 does not react in a specific fashion with $BrAcT_4$ or -T_3; *i.e.*, it is not blocked by substrate, and inactivation of D2 by $BrAcT_4$ requires concentrations 1000-fold higher than those effective for D1, suggesting that this is due to nonspecific effects of these compounds (110, 201).

Further support for this conclusion comes from studies with a human mesothelioma cell line (MSTO-211H) expressing approximately 40-fold higher levels of human *Dio2* mRNA than mesothelial cells (236). These cells have the highest D2 activity reported to date in a human tissue. This high D2 expression permitted the unequivocal demonstration that endogenous D2 is a selenoprotein encoded by the *Dio2* gene (223). D2 activity is highly Se dependent. Se depletion for 24 h reduces basal D2 activity by approximately 4-fold, whereas Se supplementation increases D2 activity by approximately 30-fold in a dose- and time-dependent fashion. In addition, an antiserum prepared against a peptide deduced from the human *Dio2* mRNA sequence precipitates a ^{75}Se protein of the predicted 31-kDa size from ^{75}Se-labeled MSTO-211H cells. The intensity of this ^{75}Se-labeled D2 band correlates with D2 activity and behaves as predicted from a number of studies using transiently expressed protein. Finally, mice with a targeted inactivation of the *Dio2* gene express no D2 activity in any tissue tested, including the pituitary, cerebral cortex, and BAT in the euthyroid or hypothyroid state (see *Section VII* and Ref. 24).

The cDNA encoding rat p29 has recently been reported (237). This was cloned from cAMP-induced rat astrocytes, but its presence or absence in tissues expressing D2, such as pituitary or BAT, was not reported. Also not discussed was the 92% identity of the deduced amino acid sequence of p29 to the COOH-terminal 275 amino acids of the mouse Dickkopf-3 (thick head) protein (GenBank accession no. AAF02680). The Dickkopf proteins (Dkk-1–4) are secreted glycoproteins that antagonize Wnt proteins, which are involved in embryonic dorsoventral patterning (238, 239). A role for p29, which appears to be a rat Dkk-3 homologue, in thyroid hormone metabolism remains to be defined.

2. Subcellular localization and topology. Early attempts were made to determine the subcellular localization of D2 in rat cerebral cortex using cell fractionation and activity measurements. D2 activity was associated with membrane fractions, but differences in the localization of PTU-sensitive and -insensitive 5′ deiodinase activities could not be elucidated (240). The availability of the D2 cDNA allowed more specific studies of the intracellular distribution of transiently expressed, catalytically active human D2 labeled with a FLAG epitope. The transiently expressed D2 is an integral membrane protein, and protease protection assays suggest that the NH_2 terminus remains in the ER lumen, whereas the COOH-terminal portion is in the cytosol (92). Immunofluorescent confocal microscopy of FLAG-D2-transfected HEK293 or neuroblastoma cells shows that transiently expressed human D2 colocalizes with glucose-regulated protein 78 (GRP78)/BiP, an ER resident protein, whereas FLAG-D1 is localized in or near the plasma membrane (Fig. 6). Endogenous D2 also colocalizes with GRP78/BiP in the MSTO-211H cells (223). This indicates that intrinsic protein sorting signals determine the differential subcellular localization of D2 and D1. Although the studies of D1 subcellular localization were performed with transiently expressed protein and need to be confirmed with primary antibodies, these different subcellular localizations can explain the ready access of T_3 generated from T_4 by D2, but not D1, to the nuclear compartment, a phenomenon noted in the earliest studies of physiology of these two enzymes (9, 94, 241).

3. Enzymatic properties and structure-activity relationships. D2 activity was first identified in pituitary as a PTU-insensitive T_4 5′-monodeiodinase (9, 167, 203). Later results showed that it has a low K_m for T_4 (~2 nM), about 3 orders of magnitude lower than that of D1 under similar *in vitro* conditions (10). rT_3 is also an excellent substrate for D2 although slightly less favored than T_4 (V_{max}/K_m ratios are 0.30 nM·min/pmol·mg protein for T_4 and 0.14 nM·min/pmol·mg protein for rT_3 (110). Deiodination by D2 requires an endogenous reducing cofactor. Its identity is not known, but DTT works efficiently *in vitro*. Deiodination by D2 follows sequential reaction kinetics, suggesting that both the substrate and a thiol must combine with the enzyme before the reaction takes place (10). D2 activity is 100-fold less sensitive to inhibition by GTG and iodoacetate compared with D1 (10, 242–244). In contrast, it has been previously shown that D2, like D1 and D3, is inhibited competitively by iopanoic acid (245). D2 is insensitive to PTU and 100-fold less sensitive to GTG than D1, the latter inhibition being noncompetitive (110). Other studies of the recombinant frog, rat, mouse, and chicken D2 proteins show similar characteristics (16, 17, 19, 20).

A Cys-for-Sec substitution at position 133 of human D2 has a major effect on its *in vitro* kinetic properties. The $K_m(T_4)$ increases 500- to 1000-fold in contrast to the increase of only 3-fold for the $K_m(rT_3)$ of the Sec126Cys D1 mutant. This

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suggests that the Sec133 residue may have an important structural role in D2. A Sec133Ala exchange inactivates the enzyme (189). The sensitivity of the Cys133 mutant human D2 to inhibition by GTG is approximately 100-fold lower than of the wild type, similar to the previous findings with D1 (107). Both the Sec133Cys and Sec133Ala human D2 mutants are expressed at levels approximately 100-fold higher than wild type, due to the increased translation efficiency of the mutants (189). The half-life of the transiently expressed Cys133 D2 is about 2 h in HEK293 cells, similar to that of the wild type. As would be predicted from the high K_m, the mutant requires 1000-fold higher rT_3 concentrations to accelerate D2 proteolysis (see *Section III.B.5* and Refs. 189 and 192).

As mentioned, transiently expressed ^{75}Se human D2 appears as a doublet, suggesting that COOH-terminal UGA acts either as a STOP or as a Sec codon (110). When the second UGA of human D2 was converted into a UGC coding for Cys or for UAA, an unambiguous STOP codon, the deiodination properties of human D2 were identical, indicating that the second Sec and the following seven amino acids are not critical for its function (202).

4. Regulation of D2 synthesis. Early data suggested that the *Dio2* gene was regulated via a cAMP-mediated pathway. Cold exposure increases D2 mRNA and activity in BAT, and α1- or β-adrenergic blocking agents abolish this effect (206, 246). In isolated brown adipocytes, the increase of D2 activity during catecholamine treatment is actinomycin D sensitive (247–249). In addition, D2 activity in BAT is induced by norepinephrine (NE), isoproterenol, insulin, and glucagon and is inhibited by GH (250, 251). cAMP increases D2 activity in rat astroglial cells (204, 252) as does both nicotine and cGMP (200, 253). D2 mRNA or activity is elevated in human thyroid tissue from patients with TSH- or Graves' IgG-stimulated thyroids, and forskolin increases D2 mRNA in dispersed human thyroid cells (195, 201).

Thus, it is not surprising that the human, rat, and mouse *Dio2* 5′-FR contain a CRE approximately 90 nt 5′ to the first TSS (Fig. 12 and Refs. 195, 197, 198, and 254). The human *Dio2* promoter activity increases 10-fold in response to the cotransfected α-catalytic subunit of PKA. Mutation of this element abolishes the effect and decreases basal expression by approximately 90% (195). The dopamine and cAMP-regulated phosphoprotein DARPP-32, a phosphatase inhibitor that can potentiate the phosphorylation of CRE-binding protein, is also present in tanycytes but not in pituitary cells (216). This enzyme may be involved in tissue-specific cAMP-mediated regulation of the *Dio2* gene.

Although there is high D2 mRNA in human thyroid, no D2 mRNA or activity is present in the FRTL-5 rat thyroid cell line, and D2 mRNA in adult rat thyroid is very low and activity is undetectable (198, 201, 255). The thyroidal expression of the human *Dio2* gene is under the control of TTF-1, a homeodomain-containing transcription factor, but is not affected by Pax-8 (198). The two TTF-1 binding sites of human *Dio2* at −235 and −620 are not present in the rat *Dio2* gene despite an overall 73% cross-species homology. This may be the explanation for the very low expression of D2 mRNA and activity in the rat thyroid (Fig. 12).

Phorbol ester treatment of cultured human thyroid or rat glial cells causes strikingly different responses in D2 mRNA levels. In thyroid cells, D2 mRNA decreased by 50%, whereas in glial cells a 10-fold induction was found (219, 256). Recently the activator protein-1 mediated suppression of D2 in the rat pineal gland has also been demonstrated using transgenic rats expressing a dominant negative *fos*-related antigen 2 (257). Mutation of the activator protein-1 site of the human *Dio2* 5′-FR up- regulates the D2 promoter by 2-fold in COS-7 cells, indicating that this site can suppress *Dio2* promoter activity in this cell type (198). This may explain the phorbol ester-induced decrease in D2 activity in thyroid cells. However, neither the wild-type human *Dio2* 5′-FR nor its AP-1 mutant responded to phorbol ester. The exact mechanism for the PKC-mediated regulation of *Dio2* remains to be determined.

Thyroid status controls D2 activity both at the pre- and posttranslational levels (245, 258, 259). Deiodination of T_4 increases in the cortex of hypothyroid rats, and hypothyroidism elevates D2 mRNA in the brain (17, 19, 215, 260, 261). Treatment of hypothyroid rats shows that T_3 decreases D2 mRNA, whereas T_4 primarily decreases D2 activity, indicating that, *in vivo*, T_3 and T_4 can exert their suppressive effects on D2 activity by pre- and posttranslational mechanisms, respectively (261). T_3-induced D2 mRNA suppression is transcriptional, because 100 nM T_3 does not affect the short (~2-h) D2 mRNA half-life, and this is a direct T_3 effect (262). Although the presence of a negative TRE in the *Dio2* 5′-FR can be inferred, it has not yet been identified. Dexamethasone and TRH modestly increase D2 mRNA in GH4C1 cells (262). In marked contrast to T_3, rT_3 reduces D2 activity but does not affect D2 mRNA levels, indicating that its regulation of D2 is completely posttranslational (262).

As mentioned earlier, the nocturnal increase in pineal gland D2 activity induced by an endogenous β-adrenergic mechanism correlates precisely with similar changes in D2 mRNA (209, 263). D2 mRNA and activity are also increased severalfold by hypothyroidism in somatosensory regions of the brain of postnatal rats, providing protection against the deleterious effects of insufficient T_3 availability during brain development (264). An effect of stress and traumatic brain injuries to increase D2 activity in the CNS has also been reported (265, 266).

5. Regulation of D2 degradation. Intracellular regulatory pathways can be modified by selective proteolysis of key rate-limiting enzymes. This process is frequently mediated by the proteasome system in which different metabolic signals stimulate ubiquitin (Ub) conjugation of target proteins and subsequent selective uptake and proteolysis in proteasomes (267, 268). D2 is a key protein in a homeostatic system that controls the intracellular concentration of T_3. D2 has a very short activity half-life (<1 h) that is further accelerated in cells exposed to its substrates, *i.e.*, T_4, rT_3, and even high concentrations of T_3 (245, 260, 269–273). MG132, a proteasome uptake blocker, stabilizes endogenous D2 activity in GH4C1 cells, even after protein synthesis is blocked by cycloheximide (191). The potency of D2 substrates to induce loss of activity mirrors the enzyme's affinity for each substrate, even if changes in affinity are induced by site-directed mutagenesis of the active center, such as in the human D2

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Sec133Cys mutant. Furthermore, substrate-induced acceleration of protein degradation is lost in a human D2 Sec133Ala mutant, which is not catalytically active (192, 245, 270). Both studies suggest that enzyme-substrate interaction must occur to induce D2 proteolysis. Moreover, the loss of activity is blocked by MG132, indicating that substrate-induced changes in D2 molecule accelerate its processing by the proteasome (191).

Direct evidence of D2 ubiquitination was obtained in ts20 cells, a Chinese hamster ovary cell line containing a temperature sensitive Ub-activating enzyme, E1. At the restrictive temperature that inactivates E1, D2 activity and protein levels are stabilized, even when protein synthesis is inhibited or D2 substrates are present. Ub-D2 conjugates are high-molecular-mass proteins (100–300 kDa) that are easily identified by Western blotting in cells transiently expressing FLAG-tagged D2 (Fig. 14). As expected, Ub-D2 conjugates are increased by exposure to D2 substrates or treatment with MG132 and decreased if E1 activity is blocked in ts20 cells. Because D2 activity correlates with the levels of D2 and not Ub-D2, it is likely that D2 is inactivated by ubiquitination. Interestingly, under the same conditions, D1 is not ubiquitinated in agreement with the long (>12-h) D1 half-life (Fig. 14 and Ref. 188).

Ubiquitination and proteasomal degradation of D2 are likely to originate at the COOH terminus, which is exposed to the cytosol. This is based on the finding of large amounts of Ub-D2 conjugates firmly associated with the ER membranes (188). In addition, fusion of the FLAG sequence to the COOH terminus of D2 not only prolongs its half-life but also increases the size of the Ub-D2 pool 20- to 30-fold when transiently expressed in HEK293 cells, whereas no change in half-life is observed if the FLAG tag is fused to the NH_2 terminus (188). It is likely that the accumulated Ub-D2 can be recycled by the action of Ub isopeptidases, explaining the prolongation of activity half-life by MG132. This implies that the D2 and Ub-D2 pools are normally in a dynamic equilibrium that shifts toward active D2 when T_4 falls or proteasomal uptake is blocked, or toward the formation of inactive Ub-D2 conjugates when cells are exposed to substrate (Fig. 15).

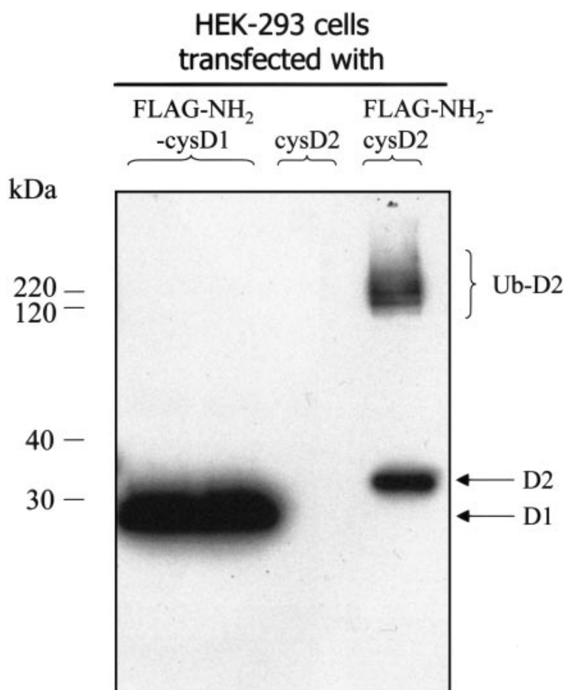

FIG. 14. Western blot of Sec126Cys D1 and Sec132Cys D2 FLAG fusion proteins transiently expressed in HEK293 cells. HEK293 cell lysates were resolved in a 12% SDS-PAGE and processed for Western blot using anti-FLAG antibody. Both enzymes are indicated by *arrows*. The D2-ubiquitin conjugates are high-molecular mass bands (100–300 kDa) as indicated (Ub-D2). Cells transiently expressing cysD2 were used as a negative control. [Reprinted with permission from B. Gereben *et al.*: *Mol Endocrinol* 14:1697–1708, 2000 (188). © The Endocrine Society.]

C. Type 3 iodothyronine deiodinase (D3)

D3 is the third enzyme involved in reductive deiodination of thyroid hormones. It is the major T_3- and T_4-inactivating enzyme because D1 (see *Section III.A*) has a weak capacity to remove iodine from the inner ring (274). D3, which exerts almost exclusively IRD activity, catalyzes the conversion of T_4 to rT_3 and the conversion of T_3 to 3,3′-T_2, both of which are biologically inactive (Fig. 1). That the products of IRD of T_4 and T_3 are ineffective in supporting thyroid hormone-dependent gene expression is illustrated by the severe hypothyroidism in patients with D3 overexpression in hepatic hemangiomas despite the markedly elevated rT_3 (see *Section VI*) and the blockade of metamorphosis in *X. laevis* tadpoles overexpressing D3 (275, 276). This enzyme contributes to thyroid hormone homeostasis by protecting tissues from an excess of thyroid hormone. It was identified in the monkey hepatocarcinoma cell line (NCLP6E), and the first extensive physiological studies were performed in the rat CNS (4, 5, 277, 278). In addition to the CNS, D3 is present in rat skin and placenta and the pregnant rat uterus, as well as in human

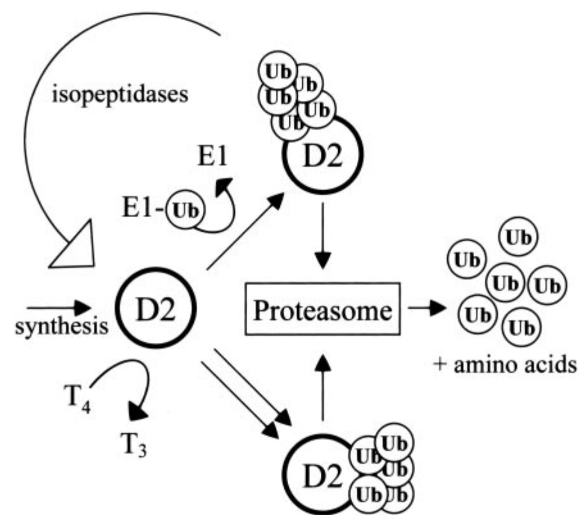

FIG. 15. Proposed model of D2 ubiquitination and degradation by proteasomes. D2 is synthesized and remains as resident protein in the ER. During its normal turnover, D2 is ubiquitinated. Catalysis accelerates ubiquitination and eventual degradation. Deubiquitination by isopeptidases is possible, particularly under conditions where the proteasomal degradation is impaired.

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embryonic liver (172), although the highest activity found to date is in human hemangiomas (276). In amphibians, D3 plays a critical role in development (279). Indeed, it is present in *R. catesbeiana* tadpoles from premetamorphosis to the onset of the metamorphic climax, after which it declines to barely detectable levels. During embryogenesis, D3 is critical for thyroid hormone homeostasis, because excess or premature exposure of the embryo to adult thyroid hormone levels can be detrimental and can result in malformations, altered growth, mental retardation, and even death. As demonstrated in several fetal and neonatal animal models, D3 expression is highly regulated in tissue-specific patterns, which are likely to be critical to the coordinated regulation of thyroid hormone effects on development (see *Section V*).

1. Gene structure, chromosomal localization, mRNA and protein characteristics, and tissue distribution.

a. Gene structure and chromosomal localization. The *Dio3* gene is found on human chromosome 14q32 and mouse chromosome 12F1 (280). Genomic cloning shows a unique feature of the *Dio3* gene when compared with D1 and D2: no introns are present in the mouse or human D3 genes (281). In this regard, D3 can be included among those rare genes in the eukaryotic kingdom (6% of total) that have no introns in their genomic structure (280, 282).

b. D3 mRNA and protein. In 1994, the cDNA for *X. laevis* D3 was cloned using a PCR-based gene expression analysis (15). Subsequently, the corresponding cDNAs of many species (rat, human, chicken, tilapia) were isolated. The human D3 mRNA is 2066 nt and contains 220 bp of 5′-UTR, an 834-bp open reading frame, and a 3′ UTR of 1012 bp (283). The deduced amino acid sequence predicts a protein of 278 residues, with a molecular mass of 31.5 kDa. Hydropathy analysis reveals a hydrophobic NH_2-terminal portion consistent with a transmembrane domain analogous to D1 (Fig. 9). All D3 cDNAs identified to date include a Sec-encoding TGA codon, as well as a SECIS element in the 3′ UTR. There is a high degree of identity between the human and other species, particularly in the putative active center, where Sec is located (Fig. 2). The conservation of this enzyme from *X. laevis* tadpoles to humans implies that its role in regulating thyroid hormone inactivation during embryological development is essential. Although the 2.3-kb band is the major mRNA in most tissues, at least four differently sized mRNAs from the rat CNS hybridize with the D3 cDNA, and dramatic changes in the relative intensity of these occur depending on thyroid status (284). Because the structure of the rat D3 gene has yet to be clarified, it is not known whether the differences in transcript sizes are due to the use of different poly(A) adenylation signals or degrees of polyadenylation.

c. Tissue distribution. IRD in general and D3 activity in particular have been described in various tissues in a number of animal species. However, most studies have been conducted with the rat model. In the adult rat, D3 is found predominantly in the CNS, skin, and placenta, whereas in the neonatal rat, skeletal muscle, liver, and intestine also express this protein (4, 150, 174, 285–287). D3 activity is also found in human fetal liver but disappears toward the end of gestation (172). In particular, using *in situ* hybridization analysis, D3 mRNA was identified throughout the brain in the adult rat CNS, with high focal expression in the hippocampal pyramidal neurons, granule cells of the dentate nucleus, and layers II–VI of the cerebral cortex (284). It is noteworthy that these regions, highly expressing D3, also contain the highest concentration of TRs in the CNS and have critical roles in learning, memory, and higher cognitive functions (288–290). Furthermore, the pattern of D3 mRNA distribution in the CNS changes during the early stages of development. At postnatal d 0, D3 is selectively expressed in the bed nucleus of the stria terminalis, the preoptic area, and other areas related anatomically and functionally to the bed nucleus of the stria terminalis such as the central amygdala, all of which are areas involved in the sexual differentiation of the brain (291). D3 expression in these areas was transient and was no longer observed at postnatal d 10. The overall pattern of rat brain D3 distribution strongly suggests that D3 is primarily expressed in neurons but it is also present in primary astroglial cultures (292–294). Recently, D3 activity and mRNA has been identified in infantile hemangiomas at levels up to 7 times those in placenta. Depending on the size of the tumor, it can cause severe hypothyroidism (see *Section VI*; Ref. 276).

D3 activity has also been described in the fetal rat retina, and in lesser amounts, in the adult eye (295). Recently, a pivotal D3 role has been discovered for retina differentiation in *X. laevis*, in which the localized expression of D3 in the dorsal ciliary marginal zone (CMZ) cells accounts for the asymmetric growth of the frog retina (Ref. 296; see *Section V*). In addition to the CNS, D3 is highly expressed in the skin of the adult rat (174, 297). Skin contains the highest rT_3 content of any tissue in the adult rat, suggesting that the high levels of D3 activity observed in skin homogenates accurately reflect the activity of this enzyme *in vivo* (297). However, D3 is not present in human neonatal keratinocyte (222).

D3 is expressed at high levels in the placenta of rat, guinea pig, and human and is by far the predominant deiodinase present in this tissue (285, 298–301). The pregnant rat uterus also expresses extremely high levels of D3, initially in decidual cells and later in the single-cell layer of the epithelium (302). D3 is at its highest levels (~500 fmol/min·mg protein) at the implantation site, nearly double the highest values obtained for any placental tissue (Fig. 16).

2. Subcellular localization and topology. An hydropathy analysis of human D3 revealed an hydrophobic NH_2-terminal portion (amino acids ~10–35) conserved in all species consistent with a transmembrane domain (Fig. 9). The microsomal localization of D3 activity, similar to D1 and D2, indicates that D3 is also an integral membrane protein, resistant to extraction from microsomal membranes by high pH (76, 303). The topology and subcellular location of D3 has not been reported.

3. Enzymatic properties. Both *in vitro* and *in vivo* analyses have demonstrated that D3 catalyzes the IRD of T_4, T_3, and 3,3′-T_2 but, interestingly, not of the corresponding sulfated iodothyronines (304). D3 from rat cerebrocortical microsomes exhibits a K_m for T_3 of 6 nM and a somewhat higher value for T_4 (37 nM). The value for T_3 is similar to that obtained for the recombinant *X. laevis* and human D3, *i.e.*, 1 nM and 12 nM,

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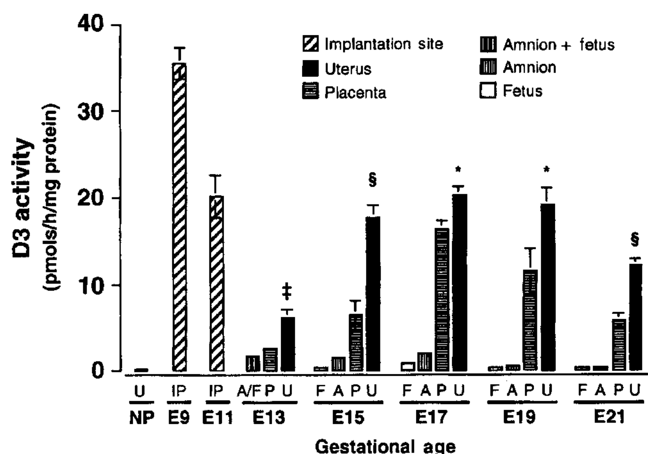

FIG. 16. D3 activity in the implantation site, uterus, placenta, fetus, and amnion at different stages of gestation in the rat. All values represent the mean ± SE of four samples harvested from the same pregnant dam at each gestational age. *, $P < 0.05$; ‡, $P < 0.01$; §, $P < 0.001$ uterus *vs.* placenta. A, Amnion; A/F, amnion plus fetus; F, fetus; IP, implantation site; NP, nonpregnant; P, placenta; U, uterus. [Reprinted with permission from V. A. Galton *et al.*: *J Clin Invest* 103: 979–987, 1999 (302).]

respectively (15, 110, 283). Early studies revealed that maximal D3 activity required a high (50 mM) DTT concentration, but the endogenous cofactor(s) has not been identified (4). D3 is insensitive to PTU inhibition, with no effect being observed up to 1 mM at varying DTT levels. GTG is a competitive inhibitor of T_3 5 deiodination, with an apparent K_i of 5.2 μM, 1000-fold greater than that for D1-catalyzed rT_3 deiodination (30, 283). D3 is relatively sensitive to inhibition by iodinated radiographic contrast agents such as iopanoic acid. Like that of D2, the deiodination reaction catalyzed by D3 follows a sequential kinetic pattern, in contrast with the ping-pong pattern of D1 (305).

As mentioned, BrAc[^{125}I]T_3 is an excellent affinity label for D1 (Fig. 8 and Refs. 25 and 103). Although early attempts to label D3 in rat placenta and brain microsomes were unsuccessful (76), recombinant human D3 can be covalently labeled with BrAc[^{125}I]T_3, appearing as a 32-kDa protein in the sonicates of transfected cells (283). Similar to D1, BrAc[^{125}I]T_3 labeling of D3 is blocked in a K_m-dependent fashion by D3 substrates, indicating that this labeling requires access to the active site. Consistent with this, GTG, a potent inhibitor of D1 BrAc[^{125}I]T_3 labeling, is much less efficient in blocking labeling of D3, with 0.2 mM GTG required for a 50% reduction. On the other hand, between 1 and 5 μM GTG are sufficient to inhibit D3 activity. This suggests that the access of $BrAcT_3$ to the binding site of D3 is favored over that of GTG. Thus, GTG is a weak competitive inhibitor of D3, suggesting that the conformation of the substrate binding site is not favorable to the entry of this compound.

4. Regulation of D3 synthesis.

a. Thyroid hormone. Parallels between D3 activity and thyroid status have been demonstrated in several species, although the underlying molecular mechanisms in mammals remain obscure. The cloning of the *X. laevis* D3 cDNA provided the first direct evidence that the *Dio3* gene is positively regulated by thyroid hormone. In *X. laevis* tadpoles, D3 is markedly and rapidly stimulated by T_3 before the metamorphic climax (82). Early studies (4) revealed that, in rats, D3 activity is increased in hyperthyroidism and decreased in hypothyroidism throughout the CNS (4). The availability of the rat D3 cDNA allowed *in situ* hybridization histochemical studies of the effect of thyroid status on D3 gene expression within the CNS. In all regions where D3 mRNA is present, its level increased 4- to 50-fold from the euthyroid to the hyperthyroid state, with the cerebellum showing the greatest increase. D3 mRNA is not detectable in Northern blots of hypothyroid brain (284). It is unknown whether the dramatic increase of D3 mRNA after short-term T_3 treatment reflects T_3-induced increases in gene transcription, mRNA stabilization, or a combination of these factors. In *X. laevis*, this effect is direct, *i.e.*, not blocked by cycloheximide. D3 promoter analysis conducted on the human and rat D3 promoters shows a positive regulation by T_3, although the magnitude of this regulation is modest compared with the effect of thyroid status on D3 activity (H. Tu, J. W. Harney, and P. R. Larsen, manuscript in preparation). Regulation of D3 activity by thyroid hormones has also been demonstrated in cultured astroglial cells. In primary astroglial cells, the addition of 10 nM T_3 (or T_4) to the culture medium caused a slow increase in D3 activity, which reached a plateau in 48 h (306).

The possibility that thyroid hormones positively regulate placental D3 activity is unsettled. Positive regulation of placental D3 activity by thyroid hormones has been reported (307), although the effect was less than 2-fold and was not observed in other studies. D3 activity is not increased in the placenta of the hyperthyroid rat, unlike the situation in brain, indicating that this gene is differentially responsive to T_3 in different tissues (285, 308).

b. Extracellular receptor kinase (ERK)-activated pathways. In the rat astroglial cell system, factors that alter cellular processes through signaling cascades originating at the plasma membrane have been demonstrated to increase D3 activity. D3 activity is markedly and rapidly induced by 12-*O*-tetradecanoyl phorbol-13-acetate and by acidic and basic fibroblast growth factors (aFGF and bFGF, respectively), as well as by epidermal growth factor (EGF), platelet-derived growth factor, and cAMP analogs, albeit to a lesser extent (309). The stimulatory effects of 12-*O*-tetradecanoyl phorbol-13-acetate and bFGF on D3 mRNA and activity appear to be mediated, at least in part, by activation of the MEK/ERK signaling cascade (310).

D3 activity is not detected in BAT at any developmental stage or in adult rats (174). However, it can be induced by serum and several growth factors in rat brown adipocytes differentiated *in vitro*, a process that requires gene transcription and *de novo* protein synthesis (311). In brown adipocytes, maximal induction of D3 mRNA occurs after 9 h of exposure to EGF, bFGF, or aFGF. The D3 mRNA half-life is 4 h when stimulated with bFGF and increases to 12 h when serum, EGF, or aFGF is present (312). The biological significance of inducible D3 in neonatal BAT is not yet clear.

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c. RA. In cultured astroglial cells, all-*trans*-retinoic acid (5 μM) causes a marked increase of up to 200-fold in D3 activity, producing an additive effect with thyroid hormones (313).

5. Regulation of D3 degradation.

a. GH and glucocorticoids. Given the similar distribution of deiodinases in chicken, rat, and humans, a useful model in which to study D3 regulation is the chicken embryo. During embryonic development, D3 levels in chicken liver are very high (74). Peaking at embryonic d 17, they then fall by approximately 98% to allow a parallel surge in plasma T_3 toward hatching (140, 314). In this model, as in general, GH injection reduces hepatic D3 activity within 2 h. A rise in plasma T_3 levels accompanies this event, confirming a primary role for D3 suppression in the GH-generated increases in plasma T_3. The changes in D3 activity are paralleled by those in mRNA, indicating that they occur at the pretranslational level (141). This also indicates that D3 mRNA and protein in the chicken hepatocyte have a relatively short half-life. Interestingly, hGH treatment of athyrotic patients receiving exogenous T_4 increases T_3 and reduces rT_3, suggesting that similar effects of GH on D3 may occur in humans (139).

Glucocorticoids reduce D3 activity in *Rana* tadpoles causing an acute increase in plasma T_3 concentrations (315). In chicken embryos, dexamethasone injection reduces hepatic D3 activity by 90% within 30 min. Although dexamethasone has a more prolonged effect than GH, maximal plasma T_3 concentrations reach only 51% of those in the GH-treated animals. As with GH regulation of D3, glucocorticoids act at the pretranslational level; whether this decrease in mRNA is due to a reduced transcription rate or to a decrease in mRNA stability is unknown (141).

IV. Summary of the Important Similarities and Differences in the Human Iodothyronine Selenodeiodinases

A comparison of the critical characteristics of the three human selenodeiodinases is presented in Table 4. Note that under the preferred substrates for D1, the V_{max}/K_m ratios for 5′ deiodination of rT_3 and IRD of T_3S are so much higher than that for T_4 that its catalytic action with respect to these two substrates may well be its major physiological action (Fig. 1). The approximately 1000-fold lower K_m (T_4) of D2 than D1 in the context of normal free T_4 concentrations in humans of 2×10^{-11} may give this enzyme a major advantage in terms of extrathyroidal T_3 production. This can outweigh the enormously higher V_{max} of D1, as opposed to D2, with respect to T_4 (but not rT_3). However, it is important to note that these kinetic constants have been measured *in vitro* using high DTT concentrations and may not necessarily reflect deiodination activity in intact cells or tissues. As an example, mutagenesis of two conserved Cys residues in rat D1 (Cys124 and Cys194) markedly reduce V_{max} values when glutathione or a reconstituted thioredoxin cofactor system were used in an *in vitro* assay. In contrast, no impairment of deiodinating capability was noted in intact cells transiently expressing these mutants (112). Nevertheless, the importance of the approximately 1000-fold difference in the K_m for T_4 between D1 and D2 in determining the pathway for T_3 production is illustrated in a tissue such as Graves' thyroid, in which both activities are highly expressed (Table 5 and Ref. 201). In human thyroid tissue sonicate, the K_m of human D1 for T_4 is about 1000 times greater than that of D2. Because of this difference, 84% of tracer T_4 is deiodinated by D2, as evidenced by the inhibition of $^{125}I^-$ release from ^{125}I-T_4 by addition of 100 nM T_4. This T_4 concentration has no effect on 5′ deiodination of T_4 by re-

TABLE 4. Human iodothyronine selenodeiodinases

Parameter	Type 1 (ORD and IRD)	Type 2 (outer ring)	Type 3 (inner ring)
Physiological role	rT_3 and T_3S degradation; source of plasma T_3, especially in hyperthyroid patients	Provide intracellular T_3 in specific tissues; source of plasma T_3 (50%)	Inactivate T_3 and T_4
Tissue location	Liver, kidney, thyroid, pituitary (?)(not CNS)	CNS, pituitary, BAT, placenta thyroid, skeletal muscle, heart	Placenta, CNS, fetal liver, hemangiomas
Subcellular location	Plasma membrane	ER	?
Molecular mass of monomer (Da)	29,000	30,500	31,500
Homodimer	Possible	?	?
Preferred Substrates (position)	rT_3 (5′), T_3S (5)	T_4, rT_3	T_3, T_4
K_m (apparent) (M)	10^{-7}, 10^{-6}	10^{-9}	10^{-9}
Active center	Sec	Sec	Sec
Susceptibility to Inhibitors/Mechanism			
PTU	High/competitive with thiol substrate	Very low	Very low
Gold	High/competitive with iodothyronine	Low/nonspecific	Low/specific, competitive with iodothyronine
Carboxymethylation	High/competitive with iodothyronine	Low/nonspecific	?
Specific labeling	Yes	No	Yes
with $BrAcT_3$, T_4	Competitive with iodothyronine		Competitive with iodothyronine
Response to Increased T_4			
Pretranslational	↑ ↑	↓	↑ ↑
Mechanism	Transcriptional	Transcriptional	Transcriptional
Posttranslational	↓ ↓ (slow)	↓ ↓ ↓ (rapid)	?
Mechanism	Oxidation of active center	↑ ubiquitination	

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TABLE 5. Analysis of the pathways of deiodination of tracer T_4 and rT_3 in a sonicate of human Graves' thyroid tissue expressing endogenous D1 and D2, in the presence of 20 mM DTT

A. Tracer deiodination		
Iodothyronine	Fractional deiodination (% tracer/h·mg protein)	
	$^{125}I-T_4$	$^{125}I-rT_3$
Tracer only	56	49
T_4 (10^{-7} M)	14	42
rT_3 (10^{-6} M)	5.7	0.2

B. T_4 and rT_3 kinetics		
Iodothyronine	K_m (10^{-7} M)	V_{max} (10^{-12} moles/min·mg protein)
T_4 (D1–no addition)	9	12
T_4 (D2–PTU 10^{-3} M)	0.03	0.11
rT_3 (D1–no addition)	1	77

combinant D1 because it is only 10% of the K_m concentration (201). On the other hand, if $^{125}I-rT_3$ is used as substrate, only 16% of its 5′ deiodination is catalyzed by D2 and therefore inhibited by 100 nM T_4. Because the endogenous free T_4 is only approximately 2×10^{-11} M, the metabolic pathways for tracer T_4 are likely to reflect those that occur *in vivo*. Similar arguments would pertain to pituitary tissue or rat cerebral cortex, in which D1 and D2 are both expressed. A similar analysis would assign D3 the major role in IRD of T_4 and T_3 because the K_m differences between D1 and D3 for IRD of T_4 and T_3 are very similar to those of D1 and D2.

The differences in susceptibility to various inhibitors of deiodination are another important tool for distinguishing between the active sites and mechanism of deiodination, particularly of D1- and D2-catalyzed T_4-to-T_3 conversion. Although the differences in the inhibition produced by PTU are nearly absolute, with D1 being completely susceptible and D2 being completely insensitive, those with respect to GTG and carboxymethylation are, relative to D1, approximately 100-fold more sensitive (316). The specificity of these differences is further revealed in their different mechanisms (competitive for D1 *vs.* noncompetitive for D2). Labeling and inactivation by BrAc-iodothyronine derivatives is specific for D1 and D3, but these compounds do not interact with D2 in a substrate-dependent fashion (110). As mentioned, confusion was engendered with respect to the identity of D2 by the fact that BrAc-iodothyronine labels a glial cell protein that is 92% identical with Dkk-3 (see *Section III.B*) and has no deiodinase activity (231). Similarly, BrAc-iodothyronine labeling of protein-disulfide isomerase initially led to the incorrect conclusion that this protein was D1 (104, 317).

With respect to the responses of the various deiodinases to alterations in thyroid status, very good information is available on the T_3-dependent transcription of the human *Dio1* promoter, and increased mRNA levels are found in human mononuclear cells from hyperthyroid patients (84). Because of the marked similarities between the rat and human D2 promoter and 5′-FR, it is reasonable to assume that there is a repression of transcription of human *Dio2* in the hyperthyroid state but that this transcriptional effect is minor in transient expression studies, about 1.5- to 2-fold (H. Tu, J. W. Harney, and P. R. Larsen, manuscript in preparation, and Refs. 198 and 262). Likewise, the high similarity between the 5′-FRs of the human and rat D3 suggest that a positive transcriptional response to T_3 is important in the regulation of D3 expression (H. Tu, J. W. Harney, and P. R. Larsen, unpublished studies), but the magnitude of this response seems much lower than is the difference in D3 mRNA levels between euthyroid and hyperthyroid rat brain (73).

V. The Physiological Roles of the Selenodeiodinases

A. The critical role of D2 in feedback regulation of TSH secretion

The first recognition that there was a PTU-insensitive pathway for T_4-to-T_3 conversion originated with the identification of the mechanism by which T_4 rapidly reduced TSH release in the hypothyroid rat. The reduction in pituitary TSH release in this paradigm began within 15–30 min of an iv bolus of either T_4 or T_3 and was not blocked by PTU (9, 166, 167, 318). A series of studies injecting combinations of $^{125}I-T_4$ and $^{131}I-T_3$ showed that TR-bound $^{125}I-T_3$ appeared in the pituitary nuclei within 15 min of $^{125}I-T_4$ injection. This could not be explained by $^{125}I-T_3$ in the plasma and was not inhibited by pretreatment with PTU (9, 94). Subsequently it was shown that pretreatment with iopanoic acid blocked both the generation of pituitary nuclear $^{125}I-T_3$ and the biological effect of T_4 on TSH release (319). Later studies in euthyroid rats indicated that this pathway for intracellular conversion of T_4 to T_3 was also present in the CNS and BAT and contributed approximately 50% or more of the specifically bound nuclear T_3 in these tissues (190, 241, 320). This 5′ deiodinase activity was directly demonstrated to have markedly different kinetic properties, substrate specificity, and regulation from D1, even though the latter is also present in rat pituitary and cerebral cortex (10, 242).

The presence of D2 can account for the requirement for physiological levels of both T_4 and T_3 for normalization of TSH. As discussed below under iodine deficiency (see *Section V*), it can account for the increase in TSH at the early stages of iodine deficiency when only T_4, not T_3, is decreased (321). Later investigations showed that the normalization of both plasma T_3 and T_4 are required to suppress TRH mRNA in the paraventricular nucleus of the hypothalamus and to normalize TSH in thyroidectomized rats given infusions of T_3 and T_4 (322–324). It is surprising that no D2 activity is present in this portion of the hypothalamus and that it is instead concentrated in the arcuate nucleus and median eminence (214, 325). Subsequent *in situ*-hybridization studies have shown that this focal collection of D2 is actually localized in the tanycytes (213, 215, 216). Because these specialized ependymal cells have their cell bodies in the inferior portion of the third ventricle, it seems likely, although it is not yet proven, that this may be a pathway by which a signal from T_4 in the central system could be transduced to the thyrotrophs via the T_3 released from the tanycyte processes into the pituitary portal plexus.

As discussed above, D2 is negatively regulated by thyroid hormone both at a pre- and posttranscriptional level, at least in the rat and mouse. An unexpected observation, however, is that, in *X. laevis*, there is an induction of D2 in the pituitary thyrotroph by T_4 and T_3 at metamorphic climax. This paradoxical increase leads to a marked reduction in the synthesis

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of TSHβ and a fall in circulating TSH at the completion of metamorphosis (326). Interestingly, the T_4- or T_3-induced increase in D2 mRNA occurs only in thyrotrophs and not in the POMC-producing pituitary cells. The mechanism for this paradoxical positive regulation of D2 by thyroid hormones resembles that seen in brown adipocytes (see *Section V.E*; Refs. 327 and 328) and could reflect the effects of T_3-induced amplification of cAMP production in response to adrenergic or other ligand signaling.

B. T_3 homeostasis

The thyroid secretes T_4 and T_3 in a proportion determined by the T_4/T_3 ratio in Tg (15:1 in humans) as modified by the minimal thyroidal conversion of T_4 to T_3 (329). Thus, the prohormone T_4 is the major secreted iodothyronine in iodine-sufficient subjects, with the ratio of secreted T_4 to T_3 being about 11:1 (330). The bulk of the daily T_3 production occurs in various extrathyroidal tissues via 5′ deiodination catalyzed by D1 and D2. The plasma concentrations of free T_4 and T_3 are constant so that tissues are exposed to the same T_3 concentrations of plasma-free hormones. However, the free T_3 concentration in different tissues varies according the amounts of hormone transported and the activity of the tissue deiodinases. These can increase (D2) or decrease (D3) the T_3 and, consequently, the nuclear TR-T_3 complexes independently of the plasma levels of thyroid hormones. As a result, the impact of the plasma thyroid hormones on target tissues is not the same in every tissue. In liver and kidney, for example, the saturation of the TRs is normally approximately 50%, whereas in the CNS it is close to 95% (Table 6). In addition, in BAT, the levels of D2 activity and TR occupancy are dynamic and change according to the metabolic requirements of the tissue. Receptor saturation is approximately 70% when the animal is at room temperature and increases to approximately 100% during exposure to a temperature of 4 C (95, 331). Lastly, changes in tissue T_3 concentrations occur throughout development as dictated by a program that coordinates adjustments in D2 and D3 activities. The deiodinases also modulate the thyroid status of individual tissues in response to iodine deficiency, hypothyroidism, or hyperthyroidism. Cells lacking the capacity to adjust the rate of activation or inactivation of T_4 and T_3 are the most affected, as their thyroid status will be determined by the plasma free T_3 concentration. On the other hand, in cells expressing D2 and/or D3, the changes in the activity of these enzymes will mitigate the fluctuations in plasma T_4 and T_3, constituting a potent mechanism for thyroid homeostasis.

TABLE 6. Source of T_3 and fraction of TRs occupied in various tissues of the rat (190, 241, 320, 423)

Tissue	T_3 (T_3)	T_3 (T_4)	Fraction of TR occupied
Liver	35	13	48
Kidney	46	7	53
Cortex	20	77	97
Cerebellum	25	37	63
Pituitary	38	40	78
BAT			
Room temperature	33	42	75
Cold	38	64	102

$T_3(T_3)$ refers to T_3 derived from plasma and $T_3(T_4)$ that derived from D2-catalyzed T_4 5′ deiodination within the cell. Values are the percentage of the maximum binding capacity of the nuclear TR for that tissue. Complete saturation is 100%.

1. Euthyroid state—sources of plasma and intracellular T_3.

a. Thyroid hormone production and clearance rates in humans. The kinetics of T_4 metabolism are markedly influenced by the extent to which T_4 is bound to plasma proteins. T_4 has a volume of distribution of only 10 liters. Because the concentration of total T_4 in plasma is approximately 100 nmol/liter, the extrathyroidal pool of T_4 is approximately 1 μmol. The fractional rate of turnover of T_4 in the periphery is normally about 10% per day (1/2 time, 6.7 d). Thus, about 1.1 liters of the peripheral T_4 distribution space are cleared of prohormone daily, a volume that contains approximately 110 nmol of T_4 (reviewed in Ref. 2). T_3, on the other hand, has a distribution volume of approximately 40 liters, making it a predominantly intracellular hormone. T_3 is produced by two different and relatively independent processes, namely by direct thyroid secretion or during extrathyroidal 5′ deiodination of T_4. The fractional turnover rate of T_3 is about 65% per day, and consequently, the metabolic clearance rate (MCR) of total T_3 is about 24 liters/d. At a mean normal serum T_3 concentration of 1.8 nmol/liter, the daily production rate of T_3 is approximately 50 nmol. This figure is an underestimate because it is calculated based solely on sampling the plasma compartment. As discussed below, in D2-expressing tissues there is a significant contribution to intracellular T_3 derived from local T_3 generation by D2. If D3 is also expressed in such tissues, an undetermined fraction of T_3 may be degraded before it enters the plasma pool.

The relative contributions of the two sources of T_3, thyroid secretion and T_4 5′ deiodination, can be quantified by determining the T_4-to-T_3 conversion rate, which is, on average, about 30–40% (2). Hence, with a normal T_4 production rate of 110 nmol/d, approximately 40 nmol of T_3 are produced by peripheral deiodination of T_4, and the remaining 10 nmol are secreted. The limited contribution of thyroidal secretion to the daily T_3 production is in agreement with the high molar ratio of T_4 to T_3 in human Tg, about 15:1 (329). Comparison of this ratio with that of T_4/T_3 in thyroid secretion of 11:1 (110 nmol T_4 and 10 nmol T_3/d) suggests that there is a contribution of intrathyroidal T_4-to-T_3 conversion in human thyroid by D1, D2, or both (201, 332).

Extrathyroidal T_3 can derive from T_4 via two different deiodination pathways, namely D1 or D2. To quantitate the role of D1 in catalyzing the production of plasma T_3, it is informative to review the results of two studies performed in patients with primary hypothyroidism who received fixed doses of exogenous T_4 (333, 334). In these patients, PTU (1000 mg/d for 7–8 d) caused a 20–30% decrease in serum T_3. In a third study, the production of labeled plasma T_3 from T_4 was not reduced in patients given 1200 mg/d of PTU (136). Results of these three studies argue that D1-catalyzed T_3 production is not a major component of extrathyroidal T_3 production in euthyroid humans. This is not, however, the case in hyperthyroid patients, in whom the contribution from D1 is clearly higher (see *Section VI*). However, it is possible that the contribution of D1-catalyzed T_4-to-T_3 conversion is underestimated either due to inadequate PTU dosage or to

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an impairment of T_3 clearance by PTU attributable to inhibition of the D1 contribution to the IRD of T_3 (274). On the other hand, there is a significant increase in the fractional conversion of T_4 to T_3 in both hypothyroidism and hypothyroxinemia. This is typical of a D2-catalyzed pathway because the opposite would be expected for D1-catalyzed T_3 production (335–337). More recent studies emphasize the difficulties in defining in which compartments extrathyroidal T_3 production occurs in humans using tracer studies (338). Depending on the assumptions used, one can obtain estimates suggesting that as much as 81% or as little as 15% of T_3 derives from rapidly equilibrating (D1-containing) tissues, with the remaining coming from slowly equilibrating (D2-containing) compartment.

b. Thyroid hormone production and clearance rates in rats. Animal models, the rat in particular, have been widely used to study the extrathyroidal metabolism of thyroid hormones. This allows the direct measurement of tissue iodothyronine concentrations under normal and varying physiopathological situations. However, there are substantial and important differences between humans and rats with respect to thyroid economy. These differences are not always appreciated so that results obtained in rats are sometimes inappropriately applied to humans.

The kinetics of T_4 metabolism in the rat are less influenced by plasma proteins, due to the weaker binding of T_4. The T_4 distribution volume is relatively larger than in humans, approximately 21 ml/100 g. Based on the plasma T_4 concentration of approximately 44 nmol/liter, the calculated extrathyroidal pool of T_4 is about 900 pmol/100 g. The fractional rate of turnover of T_4 is about 4.5% per hour (1/2 time, 11 h), resulting in a daily T_4 production rate of approximately 1 nmol/100 g of body weight. T_3, on the other hand, has a distribution volume of approximately 210 ml/100 g, which indicates the existence of one or more large extravascular pools. The fractional turnover rate of T_3 is very rapid, about 12% per hour, and consequently, the MCR of total T_3 is about 25 ml/h. At a mean normal serum T_3 concentration of 750 pmol/liter, the daily production rate of T_3 is approximately 415 pmol/100 g of body weight. In the rat, about 20–25% of secreted T_4 is 5′-deiodinated to yield T_3 (6). Hence, with a normal T_4 production rate of 1 nmol/d, 225 pmol of T_3 are produced by peripheral deiodination of T_4, and the remaining 190 pmol are secreted directly from the thyroid gland, a much larger contribution to T_3 production than in humans (40% *vs.* 20%). This is the major reason it is not possible to obtain normal tissue T_3 in all rat tissues solely by administration of T_4 (339, 340). As in humans, comparison of the molar ratio of T_4 to T_3 in rat thyroids (8:1; Ref 341) with the estimated T_4/T_3 ratio of 5:1 in thyroidal secretion (1000 pmol/d of T_4 to 190 pmol/d of T_3) indicates a small contribution of thyroidal T_4-to-T_3 conversion via D1 to the daily T_3 production in the rat.

The relative contributions of D1 and D2 pathways to whole-body T_3 production can be assessed more accurately in rats than in humans. In euthyroid rats treated chronically with high doses of PTU to inhibit D1, the T_4-to-T_3 conversion rate is reduced by 50% (6). Accordingly, in T_4-treated thyroidectomized rats, treatment with PTU results in a 50% decrease in plasma T_3 (167). This is similar to the conclusions of more sophisticated compartmental analyses of T_4-to-T_3 conversion rates in rats if we assume the T_4-to-T_3 conversion in the rapidly equilibrating pool occurs via D1 and the more delayed conversion by D2 (169). Taken together, these data indicate that D1 catalyzes about half of the daily extrathyroidal T_3 production from T_4 in the rat. Even though we do not have an accurate proportion of extrathyroidal T_3 production catalyzed by D1 in humans, the figure of 50% in rats is significantly higher than the minimal estimate of approximately 25% in humans from above-mentioned PTU studies.

There are several implications of the above calculations. Based on the data available, D1-catalyzed T_4 5′ deiodination does not appear to be the major extrathyroidal source of T_3 in the euthyroid human. This concept was eclipsed for some time because D2 activity in adult humans was believed to be restricted to the CNS and pituitary. The recent identification of D2 mRNA and activity in human skeletal muscle and heart would argue for a more important role for D2 in daily extrathyroidal T_3 production than is customarily assumed (110, 235). Second, the fact that only approximately 20% of plasma T_3 in humans comes from thyroidal secretion, as opposed to about 40% in rats, has made it more feasible to achieve physiological replacement of both T_3 and T_4 in humans with levothyroxine alone than is the case in the rat (Fig. 17).

c. Intracellular T_3 homeostasis. Plasma T_3 equilibrates rapidly with most tissues because thyroid hormones readily cross the plasma membrane by stereo-specific processes that depend on several transporters and are energy dependent (342, 343).

At equilibrium, one can estimate the nuclear T_3 from the plasma T_3 concentration and the nuclear/plasma ratio of tracer T_3. The measurement of the maximum binding capacity for the TRs allows the calculation of the TR saturation, which is normally 40–50% in most tissues (344). Thus, changes in plasma T_3 during hyper- or hypothyroidism are mirrored by changes in the TR occupancy in those tissues, which determines the intensity of the biological effects of

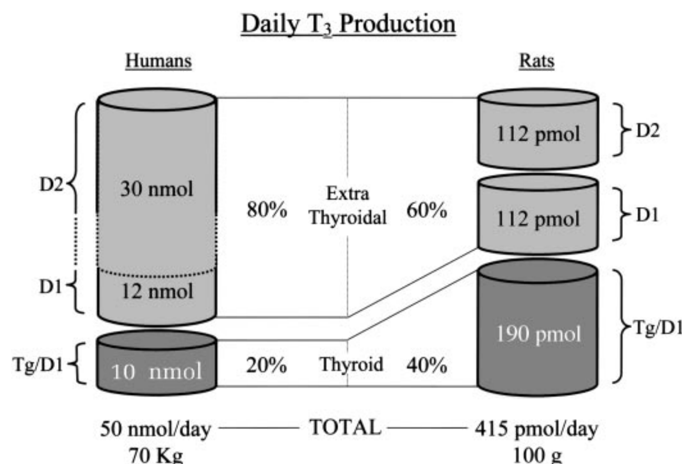

FIG. 17. Pathways of T_3 production in humans and rats. The *dotted lines in the cylinder* representing human extrathyroidal production reflect the uncertainty about the exact contributions of D1 and D2 to this pool. Values given are based on the studies cited in the text. Values for rats are normalized to 100 g body weight.

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thyroid hormones. However, in selected tissues, especially pituitary gland, brain, and BAT, there is an additional source of T_3 contributed by intracellular T_4-to-T_3 conversion (9, 345). This has been termed $T_3(T_4)$ to differentiate it from $T_3(T_3)$, the cellular T_3 derived directly from plasma. These tissues contain D2, and the T_3 generated by D2-catalyzed T_4 deiodination supplements that from plasma as though it were derived from a kinetically different pool. As a result, TR occupancy is much higher (70–90%), and 50–80% of this TR-bound T_3 is $T_3(T_4)$ (Table 6 and Refs. 94, 241, and 320). These differences have been confirmed using constant infusions of tracer T_3 and T_4 (346–349) and direct quantitation of nuclear T_3 by RIA (350). Several tissues (*e.g.*, liver and kidney) in which plasma T_3 is the only source of nuclear T_3 express D1. As discussed in *Section III*, confocal microscopic studies of transiently expressed protein suggest that D2 is located in the ER in the perinuclear region, a cellular compartment that could have preferential access to the nucleus. D1, however, has a ring-type distribution in the periphery of the cell, typical of a plasma membrane protein (Fig. 6). The rapid exit of T_3 from the cell in D1-containing tissues and its retention in D2-containing tissues explain the 3- to 4-fold higher nuclear/cytoplasmic free T_3 ratio found in brain than that found in liver, kidney, or heart (351). The consequence of the presence of D2 is that the impact of changes in secreted T_4 on cellular T_3 can be dampened at a prereceptor level by compensatory alterations in its activity.

The role of D2 in intracellular T_3 homeostasis is well established in pituitary, brain, and BAT. However, only a short list of biological effects of $T_3(T_4)$ have been fully characterized, namely the feedback regulation of TSH, rat GH synthesis, the genes involved in adaptive thermogenesis in BAT, and various enzymes in the neonatal rat brain. In all cases, the specific biological effect correlates much better with plasma T_4 or tissue $T_3(T_4)$, than with plasma T_3. However, the wide distribution of D2 in human tissues suggests that there might be other T_3-dependent biological effects that are mediated by tissue $T_3(T_4)$. For example, nine hypothyroid patients chronically treated with sufficient levothyroxine to normalize TSH had their dose altered by 25 μg in both directions (352). These changes were reflected in the expected alterations in serum free T_4 and TSH levels, but serum T_3 concentrations were not significantly changed. Remarkably, the changes in resting energy expenditure correlated directly with free T_4 and indirectly with serum TSH, and not with serum T_3. Because approximately 45% of resting energy expenditure occurs in skeletal muscle that expresses D2, it is tempting to speculate that $T_3(T_4)$, not $T_3(T_3)$, is the major physiological determinant of energy expenditure in humans.

2. Iodine deficiency and hypothyroidism. Iodine, an essential component of thyroid hormones, is available from the ocean, and salt-water vertebrates, the first life-forms to develop a thyroid gland, are not at risk for iodine deficiency. However, iodine availability can be rate limiting in terrestrial vertebrates, including humans, depending on the proximity to the ocean and the iodine content of water and the soil. Fortunately, a multiplicity of thyroidal and extrathyroidal mechanisms has evolved to mitigate the consequences of iodine deficiency on thyroid hormone synthesis, allowing nearly 2.3 billion people to live in geographical areas with low iodine soil content (353). Accordingly, no differences in growth, O_2 consumption, or thermal homeostasis were detected in rats during iodine deficiency, despite approximately 10-fold higher TSH and nearly undetectable plasma T_4 (354, 355). Not surprisingly, however, if iodine deficiency is severe and prolonged, signs of hypothyroidism do eventually develop, with reduced O_2 consumption and reduced activity of T_3-dependent enzymes (356–358). The line between compensated iodine deficiency and hypothyroidism is difficult to define experimentally, except by such measurements, because TSH is elevated at all stages in the spectrum.

The acute onset of iodine deficiency triggers a series of physiological adaptations in the hypothalamic-pituitary-thyroid axis, similar to those observed in hypothyroidism. The teleological goal of these changes is to maintain plasma and tissue T_3 in the normal range, delaying the onset of hypothyroidism. The earliest thyroidal modification is a decrease in 3,5-monoiodotyrosine, with a consequent decrease in the thyroidal T_4 while thyroidal T_3 remains constant (359). Plasma TSH concentration also rises rapidly, increasing iodide trapping via the sodium iodide symporter, thyroid blood flow, Tg synthesis, tyrosine iodination, and Tg processing (359). These modifications intensify with time, and the thyroidal 3,5-monoiodotyrosine/3-monoiodotyrosine ratio decreases approximately 3-fold and the T_4/T_3 ratio approximately 25-fold. The latter is due to a decrease in the thyroidal T_4 content, not to an absolute increase in T_3. Likewise, the increased T_3/T_4 ratio in the serum of iodine-deficient individuals is due to hypothyroxinemia, not to an increase in serum T_3. An elevated plasma TSH, along with a pronounced fall in plasma T_4 and a virtually unchanged T_3, are the physiological hallmarks of moderate iodine deficiency as well as of the early phases of primary hypothyroidism such as that due to Hashimoto's thyroiditis (341, 359, 360).

The extrathyroidal modifications during iodine deficiency or primary hypothyroidism are more complex and involve a high degree of tissue specificity. The overall fractional conversion of T_4 to T_3 is increased in the hypothyroid patient approximately 50%, *vs.* 25% in the euthyroid state (335). These results would argue that not only is D2-catalyzed T_4-to-T_3 conversion a potential source of extrathyroidal T_3 in euthyroid humans but also that an increase in D2 is an important mechanism to preserve T_3 production in primary hypothyroidism (17, 110, 235). In fact, even when circulating T_4 is reduced by TSH suppression, there is an increase in the efficiency of T_4-to-T_3 conversion (336, 337). In rats, the fractional T_4-to-T_3 conversion rate is not substantially changed by hypothyroidism. However, extrathyroidal T_3 production shifts from being relatively PTU sensitive (~50%) to a pathway that is completely PTU insensitive (361), indicating that the relative contribution of D2-catalyzed 5′ T_4 deiodination to T_3 production has increased dramatically.

In tissues that express D2, the activity of this enzyme is increased during iodine deficiency or hypothyroidism, thus increasing the local fractional conversion of T_4 to T_3 and mitigating the decrease in total T_4 (95, 152, 264, 362, 363). This has been particularly well documented for the brain, in which D2 activity and mRNA distribution are specifically

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concentrated in the hypothalamic tanycytes and the arcuate nucleus-median eminence region (213–215). BAT shows similar adaptation mechanisms (see *Section V.E*). Because of the negative regulation of *Dio2* gene transcription by thyroid hormone (262), D2 mRNA increases in iodine-deficient animals in all subregions of the brain expressing D2. Not surprisingly, however, the increases in D2 activity are much greater than those in D2 mRNA (363), similar to what is observed in hypothyroid rats (261). This is explained by the hypothyroxinemia of iodine deficiency, *per se*, acting at a posttranslational level. The mechanism by which T_4 regulates D2 protein levels has been reviewed in *Section III* and is due to a substrate-induced increase in the rate of D2 ubiquitination, accelerating degradation in the proteasomes (188, 191, 192). Thus, when plasma T_4 falls, the D2 half-life is prolonged, resulting in an increase in the D2 protein/ mRNA ratio.

In addition to increasing the fractional T_4-to-T_3 conversion, the clearance of T_3 from the brain is reduced during hypothyroidism. This is because D3 is a T_3-dependent gene, and its activity correlates with thyroid status. Both fetal and adult rat brain respond to iodine deficiency by decreasing D3 activity, but only modest (2-fold) reductions occur (4, 15, 152, 284, 364). Despite its critical role, it was only recently demonstrated that the distribution of D3 in the CNS, like that of D2, is heterogeneous, with high focal expression in the hippocampus and cerebral cortex (284, 291). However, in specific brain subregions such as cerebral cortex, hippocampus, and cerebellum, D3 activity is decreased by 80–90%, changes of a much higher magnitude than occurs in the brain in general (152). The consequences of the fall in D3 activity are 2-fold. First, there will be an increase in the residence time of T_3 within the tissue because the rate of T_3 degradation via IRD will be reduced (365). Second, because T_4 is also a substrate for D3, relatively more of this prohormone will remain within the tissue for conversion to T_3 by D2. Particularly in tissues such as brain, in which the exchange of T_3 with plasma is slow and most of the T_3 is generated *in situ*, it is likely that fluctuations in the rate of T_3 degradation will have a greater influence on tissue levels of T_3 than will occur in tissues that are in rapid equilibration with plasma, such as liver and kidney (190). This prediction has been borne out using dual-labeling *in vivo* techniques with which the disappearance of tracer T_3 from cerebral cortex and cerebellum was found to be significantly slower in hypothyroid rats, a situation in which CNS D3 is also decreased (365).

The increased fractional production of T_3 from T_4 by D2 combined with the prolonged residence time of T_3 will mitigate the effects of severe iodine deficiency as has been demonstrated in mild to moderate hypothyroidism using tracer studies (258). These predictions were confirmed directly by measuring thyroid hormone concentrations in various regions of the CNS in iodine-deficient rats (362). As expected, tissue T_4 was markedly decreased, whereas tissue T_3 concentrations were reduced by only 50%. This illustrates the effectiveness of these compensatory mechanisms.

C. Embryonic development and metamorphosis

An appropriate thyroid hormone level is critically important for the coordination of developmental processes in all vertebrate species. During embryogenesis, thyroid hormone acts primarily to promote differentiation and thus attenuate proliferation. As a result, either insufficient levels of T_3 or the premature exposure of the embryo to adult T_3 concentrations can be detrimental and can result in abnormal development (366). As an example, exposure of the neonatal rat to excessive thyroid hormones causes accelerated morphogenesis of pyramidal neurons and their dendritic spines in the cerebrum as well as a persistent reduction in the total neuronal cell number (367). The best characterized action by which thyroid hormone influences developmental processes is via changes in gene expression initiated by the binding of T_3 to TRs (368, 369). During development in experimental animals, two deiodinases (D3 and D2) exert the major control of T_3 concentrations (174). As mentioned earlier, circulating T_3 is very low in the fetus, and during early development D3 is the predominant deiodinase expressed in most rat tissues with much higher activities than found in adults. D3 is also expressed in the human fetal liver but decreases toward the end of gestation (172). This pattern suggests that D3 plays a major role in preventing premature exposure of fetal tissues to inappropriate levels of T_3. Although this is a general concept, it may also be tissue-specific such as in the *X. laevis* retina (Ref. 296; see *Section V.C.3*). Conversely, during development D2 is expressed in most mammalian tissues over a restricted period of time. This points to a tissue-specific T_3-dependent differentiation program as has been observed during tadpole metamorphosis, in rats during neuronal and glial maturation, or in the rat cochlea (82, 212, 370, 371). Finally, D1 is generally lower during fetal development than at later stages of life (372). This would again reduce circulating T_3 concentrations.

1. Deiodinases in mammalian development. D2 expression with a precise timing is fundamental during critical periods of mammalian development. In rat brain, D2 increases rapidly after birth, reaching its highest level around d 28, and then declines, reaching adult levels by d 50 (278). Cochlea is among the organs most sensitive to thyroid hormone abnormalities, as is evident from the deafness that may be associated with congenital hypothyroidism. To complete cochlear maturation and the onset of auditory function, T_3 must be present at critical period between the late embryonic stage and the second postnatal week. So far, little is known about the mechanisms that control this temporal regulation. Analysis of cochlear homogenates from postnatal d 2–postnatal d 8 pups identify a striking D2 activity peak around postnatal d 7, which declines abruptly by postnatal d 10, a few days before the onset of hearing (Fig. 18 and Ref. 212). Relative to serum, cochlear tissue has a high T_3/T_4 ratio, supporting a role for D2 in amplifying local T_3 levels. D2 mRNA is localized in connective tissue, close to the region where dendritic and axonal projections connect with the hair cells. D2 expression was complementary to, rather then coincident with, that of TRβ, suggesting a paracrine rather than endocrine mode of signaling in cochlear tissue. This model resembles the recently proposed model in the rat brain, in which D2 is mainly expressed in astrocytes and not in the neurons that are the primary T_3 targets (215, 264).

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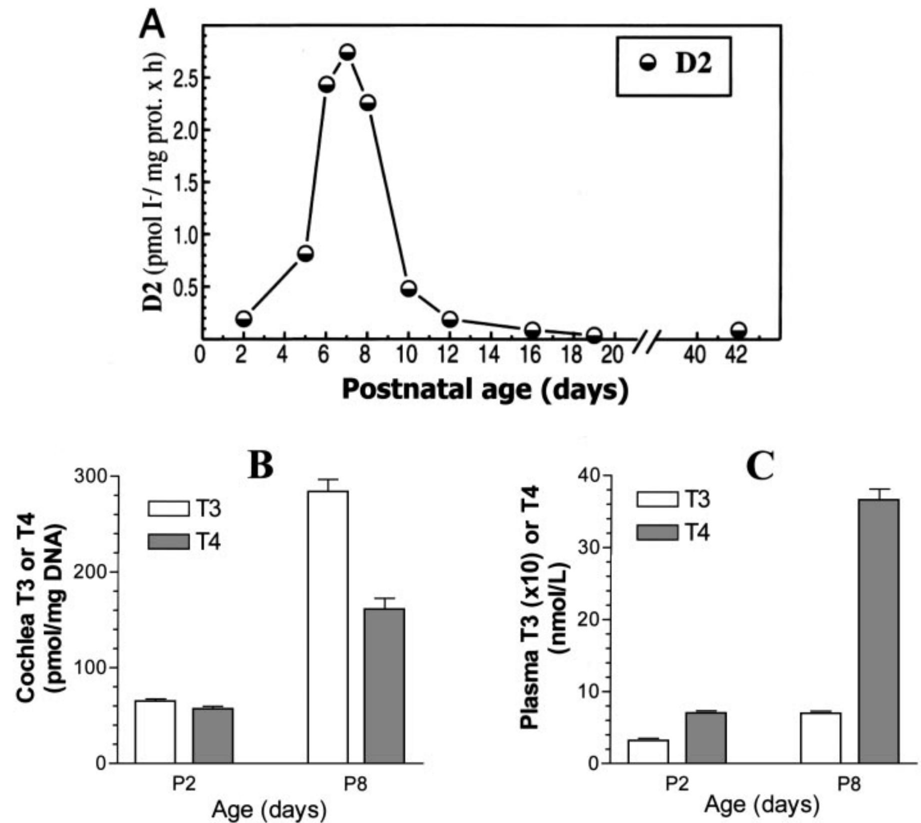

FIG. 18. A, Developmental profile of D2 activity in mouse cochlear homogenates. Values are the mean of two to six determinations. For each determination, pools of cochleae (from five to eight litter-matched pups) were assayed for D2 activity. B, T_3 and T_4 concentrations and T_3/T_4 molar ratios in cochlear tissue and serum of neonatal mice (C). Values are the means ± SEM of three to four separate determinations, each representing the pooled cochlear tissue or pooled serum of four to seven litter-matched pups of the indicated ages. [Reprinted with permission from A. Campos-Barros *et al.*: *Proc Natl Acad Sci USA* 97:1287–1292, 2000 (212). © National Academy of Sciences USA.]

2. Deiodinases in avian development. Another model that illustrates the key role of the deiodinases in fetal maturation is the chick embryo. During embryonic development, D1 activity increases progressively in chicken liver during the last week of embryonic development. On the other hand, D3 increases 2- to 3-fold from d 14 to 17 (total 21 d), to fall abruptly by approximately 98% toward the moment of internal pipping and hatching (74, 314). In this animal, there is always a positive relationship between plasma T_4 and D1 activity and, conversely, a negative correlation between plasma T_3 and hepatic D3 activity. The rapid fall in hepatic D3 activity occurs at pretranslational levels (141). This decrease is very likely due to the increases in endogenous GH and glucocorticoids, which occur just before hatching (see *Section III*).

3. Amphibian metamorphosis. The involvement of the thyroid gland in amphibian metamorphosis has been recognized for almost a century (373). *X. laevis* tadpoles, in which endogenous thyroid hormone biosynthesis is blocked by perchlorate, do not enter metamorphosis, but injection of either T_3 or T_4 restores the normal process. A similar block to metamorphosis is observed in transgenic *X. laevis* tadpoles that overexpress D3, demonstrating that high levels of this deiodinase will modulate the action of thyroid hormones *in vivo* by decreasing T_3 concentrations (275).

In developing *R. catesbeiana* tadpoles, the timing of the thyroid hormone-dependent metamorphic responses varies markedly among tissues. The coordinated development of the different organs depends on the tissue-specific expression of D2 and D3 to achieve the appropriate intracellular T_3 levels. D1 is absent in *Rana*. The profiles of D2 expression in tail, hindlimb, forelimb, intestine, skin, and eye differ markedly in both activity and mRNA levels, but it is notable that expression is invariably highest in a given tissue at the time of its major metamorphic change. Thus, in tail, which starts to resorb after climax, D2 expression is minimal before climax and then increases rapidly, whereas in limb, D2 expression is highest during prometamorphosis, the timing of differentiation for this tissue (82). This situation is different from events in *X. laevis*, in which D3 activity decreases just before metamorphic changes (275).

In *R. catesbeiana* tadpoles in which endogenous thyroid hormone synthesis was blocked with methimazole and the activities of D2 and D3 were inhibited by iopanoic acid, metamorphosis was blocked. The inhibition could be overcome by the concomitant administration of replacement levels of T_3, but not T_4 (82). These results illustrate that the expression of D2 and D3 is programmed to provide the necessary amount of T_3 at the appropriate time of development.

An example of a precisely timed local regulation of T_3 production by programmed local D3 expression occurs in the visual system of *X. laevis*. Metamorphosis in amphibians includes a remodeling of various aspects of the visual system. Eyes shift from a lateral position in tadpoles to a more rostral and dorsal location in frogs so that they may have overlapping visual fields. Retinal cells follow this shift with an asymmetrical growth in the corresponding CMZ. This asymmetrical retinal growth is thyroid hormone dependent, because it is inhibited by blocking production of T_4 and can be induced precociously by the addition of exogenous thyroid hormone (374–376). In the CMZ, a subset of dorsal cells

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express D3 starting at embryogenesis, and these are the cells that do not grow in the presence of thyroid hormone at metamorphosis (Fig. 19). In this model, it has been recently demonstrated that transgenic expression of D3 inhibited thyroid hormone-dependent proliferation of retinal cells and that dorsal retinal cells are resistant to exogenous thyroid hormone, but this resistance is abrogated by iopanoic acid (296). These results demonstrate that the localized expression of D3 is sufficient to account for the asymmetric response of the retina at metamorphosis.

Equally critical is the previously mentioned increase in D2 activity induced by T_3 or T_4 in the *X. laevis* thyrotrophs at metamorphic climax (326). This permits the high circulating T_4 characteristic of this metamorphic stage to stop TSH production, thereby terminating T_4 production at the completion of metamorphosis.

4. Other vertebrates. In late autumn, the female salmon buries fertilized eggs that will hatch into alevin, which will leave the nest and grow to fry. Fry quickly develop into parr with camouflaging vertical stripes. One to 3 yr later, the stream-dwelling parr undergo a smoltification process in which they are transformed into seawater-adaptable smolts. This metamorphic process is characterized by timely changes in metabolism, growth, osmoregulation, behavior, and olfaction (377, 378). This process is directed by a series of hormones, one of which is thyroid hormone (379). Imposing a 16-h photo period can induce the parr-smolt transformation in a 5-wk period. Plasma T_4 and T_3 peak during wk 3–4 (2- to 3-fold), returning to normal values by wk 5. The peak in T_3 is paralleled by an increase in D2 activity in liver, heart, and brain, but only D2 activity in liver correlated significantly with plasma T_3. Brain D3 activity increases progressively during smoltification, and in post-smolts, both hepatic and brain D2 and D3 activities are very low (380, 381). These studies indicate that the tissue T_3 concentration is tightly regulated in salmon by both D1 and D3. Given the thyroid sensitivity of smoltification, it is likely that the deiodinases play a fundamental developmental role in salmon.

Spontaneous metamorphosis in larval sea lampreys (*Petromyzon marinus*) depends on morphometric parameters such as body length and weight and also on thyroid hormones (382). As the larvae grow, there is a gradual rise in T_4 and T_3 serum concentrations, which peak just before the onset of metamorphosis. Both T_4 and T_3 decrease markedly at the same time as the first external changes are detected (383, 384). This decrease in thyroid hormones is required for metamorphosis, and in fact, precocious metamorphosis can be induced by perchlorate treatment or blocked by the administration of either T_4 or T_3 (382). Interestingly, this pattern is quite different from that in other vertebrates, in which there is a rapid rise in serum T_4 and T_3 during prometamorphosis with a peak followed by a decline during metamorphic climax. In the sea lamprey, D2 increases in the intestine of premetamorphic animals, is highest in stages 1 and 2, and is very low during stages 3–7 of metamorphosis. D3 is negligible until stage 3 but increases approximately 5-fold through stages 3–7, and the resulting D3/D2 activity ratio is approximately 14 at stage 6 (349, 381). These reciprocal changes of D2 and D3 seem to be part of a programmed change during metamorphosis such that D2 predominates in the early phase, whereas D3 predominates in mid- and late metamorphosis. This may contribute to the fall in plasma T_4 and T_3 typically observed after spontaneous metamorphic climax.

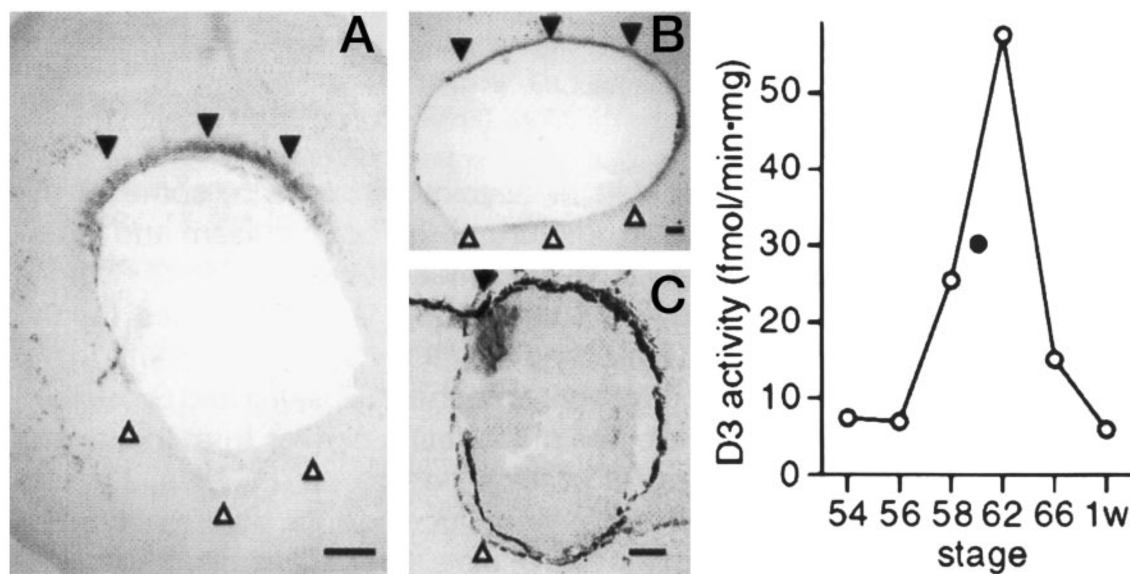

FIG. 19. D3 mRNA is expressed in the retina of *X. laevi* before and during metamorphosis. A, D3 mRNA is expressed at stage 54 in marginal cells in the dorsal third of the retina. Flatmount view. B, By stage 59, D3 mRNA has spread ventrally to over half of the margin, spreading further down the nasal half of the retina. Flatmount view; nasal is to the *right*. C, At stage 36, D3 mRNA is already expressed in the dorsal retina margin. Transverse section. D, D3 enzymatic activity in the retina (*open circles*) peaks at metamorphic climax. 1w Represents 1 wk after metamorphosis. The *closed circle* is the value obtained in the same experiment for a tail of a stage-60 tadpole. A–C, Dorsal is up, and *black-and-white arrowheads* point to the dorsal and ventral CMZ, respectively. *Scale bars* represent 100 μm in A and B and 50 μm in C. [Reprinted from N. Marsh-Armstrong *et al.*: *Neuron* 24:871–878, 1999 (296). © Elsevier Science.]

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D. Maternal-fetal physiology

The capacity to synthesize thyroid hormones does not appear until 10–12 wk gestation. However, human fetuses have thyroid hormone-occupied TR preceding the onset of active iodine uptake and secretion of hormones by fetal thyroid (385). Before the fetal thyroid gland becomes functional (around d 17–18 in rats and d 90 in humans), fetal thyroid hormones must come from the maternal circulation. Even after the onset of fetal thyroid function, they may contribute to the maintenance of fetal thyroid status. During most of the first trimester, the amniotic cavity containing the developing embryo is surrounded by the extraembryonic coelom containing the coelomic fluid, which is surrounded in turn by the placenta (386). At 6 to 12 wk gestation, the average total T_4 concentrations are 146, 0.96, and 0.02 nmol/liter in maternal serum, coelomic fluid, and amniotic fluid, respectively, suggesting a marked gradient of T_4 from mother to fetus. The gradient for rT_3 is in the opposite direction, being 3.8 and 15 times the maternal levels in the coelomic and amniotic fluid, respectively (Table 7 and Ref. 386). Also during the second and third trimesters, there are marked maternal-to-fetal gradients of free T_4 and T_3 (387, 388).

D2 and D3 activity appear in fetal tissues at midgestation, whereas D1 is not evident until later (173). Accordingly, fetal serum T_3 concentrations are quite low before 30 wk of age (see Table 7), with a modest preterm increase in fetal serum T_3 concentrations due to an increase in D1 activity. The sulfated iodothyronine concentrations are higher in the umbilical cord than in adults, and although T_3S does not bind to TRs, if local desulfation occurred, this would provide a local source of T_3 in those fetal tissues (389–392). Sulfation of T_4 and T_3 dramatically increases their IRD (inactivation) by D1. The reason for the high concentrations of sulfated iodothyronines in fetal plasma is still unknown. It is clear from these data that the pattern of circulating iodothyronines in the fetus is characterized by low levels of serum T_3 and a high rT_3 due to the combination of high tissue D3 and low D1 throughout most of gestation.

1. Placental thyroid hormone transfer is modulated by D3. The placenta is the pathway for maternal-fetal thyroid hormone transmission and can be an important determinant of the thyroid state of the fetus (Fig. 20). Placental D3 activity increases with gestational age in rats as well as in humans (285, 299, 393, 394). In the first trimester, when the placenta and the transport surface area are small, there is high specific D3 activity. At term, specific D3 activity is decreased, but because the placenta and the surface area are much larger than in the first semester, the total placental D3 activity is increased. In rats, unlike humans, placental D3 activity increases about 2-fold from d 14 until d 16 or 17, after which a decrease is observed (285, 395). Part of these differences between the human and rat could be explained by the decreased protein and DNA concentrations in rat placenta during pregnancy, as opposed to the protein and DNA in human placenta, which increase with time (396). As mentioned, placenta also contains D2; however, at all gestational ages, placental D3 activity is approximately 200-fold higher than is D2. Semiquantitative RT-PCR of the D2 and D3 genes in placentas from different gestational ages showed that there is no direct correlation between D2 activities and mRNA levels, and although D3 enzymatic activity is always higher than D2 activity, this is not true for the mRNA levels in the same samples (394).

The cellular localization is also different in placenta between D2 and D3. D2 activity is higher in the chorionic and decidual membranes of the placenta than in the amniotic membranes, whereas D3 is found mostly in trophoblasts (301). However, given the very low levels of D2 at all gestational ages, fluctuations in D2 activity are not likely to have a significant effect on fetal thyroid hormone concentrations

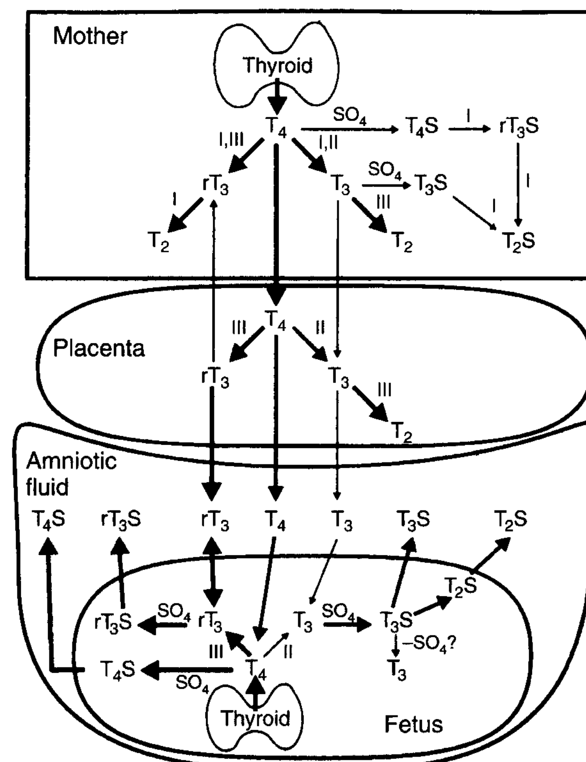

FIG. 20. Interrelations of maternal, placental, and fetal thyroid metabolism. I, II, and III denote D1, D2, and D3, respectively. SO_4 is a sulfation pathway, and $-SO_4$ is a desulfation pathway. [Reprinted with permission from G. N. Burrow *et al.*: *N Engl J Med* 331:1072–1078, 1994 (173). © Massachusetts Medical Society.]

TABLE 7. Iodothyronine concentrations in maternal and fetal serum and amniotic fluid (173)

Iodothyronine	Maternal serum*	Amniotic fluid		Fetal serum	
		20-wk	Term	20-wk	Term
T_4	12,000	250	570	3,100	11,000
T_3	200	8.6	6.6	13	49
3,3′ T_2	2.2	5.8	6.2	–	11
rT_3	24	130	69	250	270
T_4S	1.8	28	–	–	21
T_3S	2.9	6.6	–	6.6	12
rT_3S	3.8	8.6	–	–	50

Data are from Ref. 511. Iodothyronine concentrations are expressed as ng/dl and refer to total iodothyronine concentrations. *, Values are for midgestation. *Dashes* indicate that data are not available.

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but could play a role in the regulation of intraplacental T_3 generation.

The physiological significance of the high D3 in placenta is clear. Studies of the *in situ*-perfused guinea pig placenta demonstrate that T_3 is actively deiodinated to 3,3′-T_2 and a small portion is further deiodinated to generate 3′-T1 (298). In the isolated, perfused human placental lobule, little of the T_4 added to the maternal side appears in the fetal circuit. In contrast, rT_3 rises progressively on both sides. Addition of the deiodinase inhibitor iopanoic acid to the maternal perfusate completely alters the results. There is an increase in T_4 appearance on the fetal side and a significant reduction in rT_3. This is direct evidence that human placental D3 is a major factor controlling transmission of maternal T_4 to the fetus (397).

The pregnant rat uterus also expresses extremely high levels of D3, initially in decidual cells and later in the single-cell layer of the epithelium (Fig. 21 and Ref. 302). At the implantation site, as early as gestational d 9, D3 is at its highest levels (~0.5 pmol/min·mg protein), almost double the highest values obtained for any placental tissue. This finding is of particular significance, considering that TRα mRNA is not unequivocally expressed in the neural tube of the rat fetus before embryonic d 11.5 and that placenta becomes functional at embryonic d 11 (398). Throughout gestation, D3 activity remains higher in the uterus than in the placenta and is 10 times higher than in the entire fetus. D3 activity has also been detected in the amniotic fluid (302). Thyroid hormones exert a significant effect on developmental processes during embryonic life, and particularly high concentrations of T_3 are dysmorphogenic and induce structural abnormalities in the cephalic and brachial arches when given with 9-*cis*-retinoic acid (399). The elevated activity of D3 in the uterus, amnion, and placenta thus represents an effective barrier to the passage of maternal thyroid hormone. This barrier is so potent that instillation of 700 μg of T_4 into human amniotic fluid at term causes insignificant increases in the neonatal serum T_3 concentrations assessed 24 h later (400).

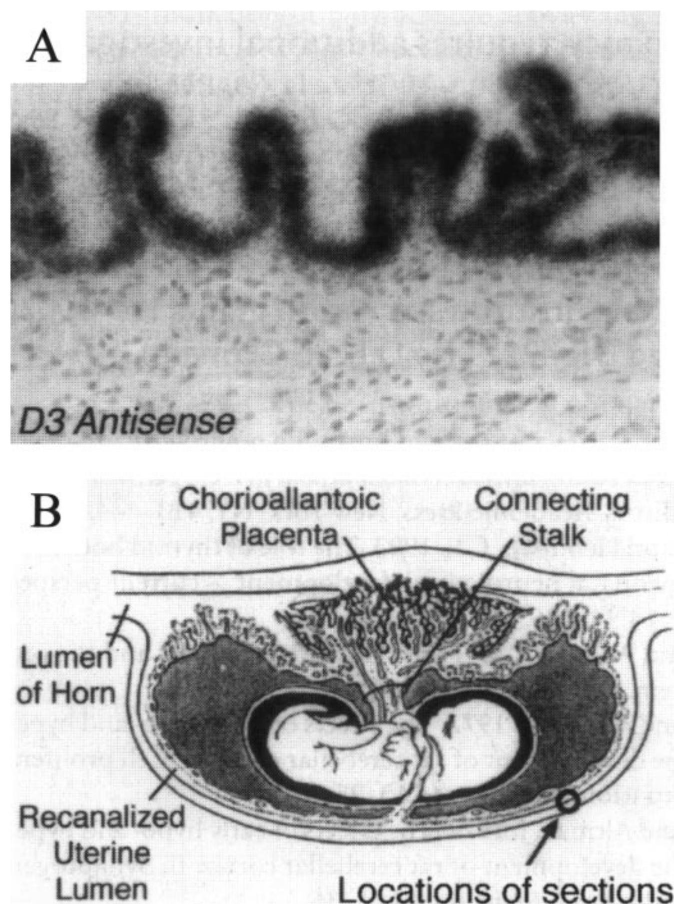

FIG. 21. *In situ* hybridization using D3 antisense and sense probes on sections of a uterus from a pregnant rat at embryonic d 19. The specimen was harvested so that the epithelial cells lining the recanalized uterine lumen lie on the outside of the specimen. A, High-powered light-field photomicrography of a section hybridized with the D3 antisense probe showing intense signal over the epithelial cells. B, Diagram of a longitudinal section through the fetal cavity and uterus of a late-stage rodent pregnancy, illustrating the approximate locations of the section in this figure. [Reprinted with permission from V. A. Galton *et al.*: *J Clin Invest* 103:979–987, 1999 (302).]

Paradoxically, despite T_4 and T_3 inactivation by uterus and placenta, neonates with congenital hypothyroidism often have little evidence of the condition at birth, suggesting significant placental transfer of maternal thyroid hormone. It has been directly demonstrated that cord blood T_4 levels in neonates with a total thyroidal organification defect are 20–50% of normal and that these decrease rapidly after birth (401). Even in severely hypothyroid newborns with markedly reduced serum T_4 levels, serum T_3 and placental D3 activities were similar to those of euthyroid newborns. This suggests that placental D3 activity is regulated by serum T_3 (401). These results indicate that a steep maternal-fetal gradient somehow overcomes the placental barrier, permitting maternal T_4 to enter the fetal circulation. Recently, substantial levels of D2 activity were found in nonpregnant rodent uterus, which were further increased during pregnancy (210). D2 activity predominates in the regions of the uterus surrounding the decidual reaction and could serve as a source of T_3 to the embryo.

The human utero-placenta unit is a sophisticated system that can regulate the amounts of transferred maternal T_4 and T_3 in relation to the age of the developing fetus and the production capacity of the fetal thyroid gland. The maternal T_4 contribution gradually decreases with time, although it is still detectable at term. This programmed deiodinase expression allows the maternal and fetal thyroid axes to function relatively independently. In this way, fetal thyroid hormones levels can be regulated primarily by fetal developmental program, whereas the maternal thyroid axis can respond to the unique needs of the mother.

E. The essential role of D2 in adaptive thermogenesis

1. Obligatory vs. adaptive thermogenesis. Hypothyroid patients are cold intolerant and may be hypothermic, whereas the opposite is observed in thyrotoxicosis. This is largely explained by the role played by thyroid hormones in thermogenesis and energy homeostasis, as evidenced by their positive influence in the BMR. This is the energy expenditure necessary to sustain minimal homeostatic functions as mea-

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sured at rest in a 12 h-fasted, fully relaxed subject kept at room temperature. Similar to an idling car engine, sustaining the BMR results in substantial heat production, termed "obligatory thermogenesis." The heat production is explained by the intrinsic thermodynamic inefficiency of energy transformation. Rather than being lost, this heat serves to increase the body temperature to one at which enzymatic reactions and biological functions operate optimally. Most of the time, however, endothermic animals function at higher rates than the BMR. This is because any physical or metabolic activity that disrupts the resting state requires extra energy and therefore accelerates ATP utilization. Obligatory thermogenesis is sufficient to sustain a core temperature of approximately 37 C only over a narrow range of ambient temperatures (26–28 C). Consequently, room temperature (21–22 C) is a significant cold stress for small mammals, including newborn humans and rodents, creating the need for additional heat to allow effective thermoregulation. This supplementary category of heat production is known as "adaptive thermogenesis," which, contrary to obligatory thermogenesis, may fluctuate rapidly in response to triggering signals. Both obligatory and adaptive thermogenesis are markedly up-regulated by thyroid hormones. This is a tremendous thyroid hormone-mediated evolutionary advantage that has allowed endothermic animals to live in and dominate virtually all environments (see Ref. 402 for review).

2. Adaptive thermogenesis in small mammals requires BAT. During cold exposure, stimuli from the hypothalamus initiate shivering and activate the sympathetic nervous system (SNS) to increase the release of catecholamines throughout the body. Shivering is the most important involuntary mechanism of cold-induced adaptive thermogenesis in adult humans and in large mammals. On the other hand, nonshivering adaptive thermogenesis is the most important heat source in small mammals, including the human newborn. This is because shivering increases peripheral blood flow and inevitably causes convective heat loss due to body oscillations and is therefore a less economical form of heat production, particularly in smaller organisms with a high surface to mass ratio. The maximum extent of nonshivering thermogenesis is inversely related to body size and, in subjects heavier than approximately 10 kg, NE-induced nonshivering thermogenesis is negligible (403).

BAT is the key organ in the cold-induced adaptive (nonshivering) thermogenesis. BAT is intensely innervated by the SNS, and its thermogenic capacity is largely due to uncoupling protein-1 (UCP1), a mitochondrial protein that short-circuits the proton gradient across the inner mitochondrial membrane, bypassing the less abundant ATP synthase and thereby uncoupling fuel oxidation from the phosphorylation of ADP (404, 405). UCP1-knockout mice are cold intolerant, illustrating the important role of UCP1 and BAT in adaptive thermogenesis (406).

In the normal adult human, the presence of isolated brown adipocytes or islands of typical BAT adjacent to blood vessels is restricted to the axillary, deep cervical, and perirenal adipose depots (407), in agreement with a minor role of BAT in large mammals. Only in patients with pheochromocytoma is there a prominent typical BAT with increased mitochondrial UCP1 (408, 409). In infants, on the other hand, the mass of BAT peaks at the time of birth, and typical multilocular brown adipocytes can be found in virtually all adipose depots of newborns, comprising almost 1% of their body weight (410). Interscapular BAT from human newborns contains significant amounts of UCP1, comparable to levels seen in cold-exposed rats (411). Even though the shivering mechanism is well developed at the time of birth, infants rarely shiver in response to cooling. This is because during the first 3–6 months of life, the shivering threshold is reduced to a lower body temperature and their metabolic rate is increased due to efficient BAT-mediated nonshivering thermogenesis (403).

3. Type 2 deiodinase is required for normal BAT function. Even though normal diet-induced adaptive thermogenesis occurs in hypothyroid rats (412), most, if not all, of the cold-induced nonshivering thermogenesis depends primarily on the synergism between catecholamines and thyroid hormones. For example, NE infusion, which normally increases total body O_2 consumption 2- to 3-fold, fails to do so in hypothyroid rats (413). Due to alterations at the various levels of the adrenergic transduction system, hypothyroid BAT is less responsive to adrenergic receptor stimulation and fails to increase cAMP normally (414–416). As a result, cold-exposed hypothyroid rats will become profoundly hypothermic and will succumb (417). The augmentation of adrenergic responsiveness by thyroid hormone, as reflected in brown fat thermogenesis and cAMP generation, is mediated almost exclusively by TRα (418).

Brown adipocytes constitute a unique example of an intricate interaction between the thyroid and the SNS. Interscapular BAT of hypothyroid rats does not respond thermogenically to NE infusion, whereas in intact rats, BAT temperature rapidly increases approximately 3 C (419). This is, in part, explained by mechanisms operating at the UCP1 gene, which is under tight control by NE and thyroid hormones. This has been extensively studied *in vivo* (419–425) in freshly dispersed (426) or cultured brown adipocytes (427). Cold exposure induces a rapid increase in UCP1 gene expression by transcriptional and posttranscriptional mechanisms (422, 424). As a result, UCP1 mRNA levels increase 3- to 4-fold after only 4 h, and mitochondrial UCP1 content increases 2- to 3-fold within 4–5 d of cold exposure. Both *in vivo* and *in vitro* studies indicate a strong synergism between T_3- and NE-generated signals to stimulate UCP1 gene transcription, culminating in an approximately 8-fold induction in just a few minutes (422, 426). The molecular basis of this synergism relies on two functional TREs and a CRE in the UCP1 gene promoter and on the proteins involved in cAMP generation (402, 428). However, after a few hours of cold exposure, the sympathetic stimulation of BAT is restrained by systemic (429) and local (430, 431) mechanisms, and UCP1 gene transcription returns to baseline values. The high UCP1 mRNA levels during prolonged cold exposure are sustained by a 4-fold increase in its half-life, a phenomenon that is thyroid hormone dependent (424, 426). T_3 plays an important role in sustaining a higher UCP1 concentration during this post-acute phase of cold exposure, and this T_3 effect can be

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detected even under conditions of minimal sympathetic activity (425).

The normal response of UCP1 to cold exposure is blunted in hypothyroid rats (420, 421, 432) and requires complete saturation of BAT TR (420). This is evident from plots of BAT mitochondrial UCP1 levels against TR saturation during acute thyroid hormone treatment of cold-exposed hypothyroid rats. From the low hypothyroid levels of TR saturation up to approximately 70%, the response of UCP1 to cold exposure is only one-fifth of that observed in euthyroid rats. As TR saturation increases further, however, the UCP1 response is augmented up to the levels seen in cold-exposed euthyroid rats (420). As with total body O_2 consumption, normalizing the UCP1 response of the hypothyroid rats with exogenous T_3 requires doses that cause systemic hyperthyroidism (423). In contrast, the same result occurs with only replacement doses of T_4. This implies an important role for T_4, *per se*, in the response to cold that results from the D2 expressed in BAT. D2-catalyzed T_4 5′ deiodination generates the additional T_3 required for adaptive thermogenesis in BAT (206). This avoids the requirement for an acute systemic increase in thyroid hormones.

A direct role for an acute increase in T_4-to-T_3 conversion in energy homeostasis was first suggested by the finding of D2 in BAT (206, 243). Stimulation of the BAT by the SNS during cold exposure or after injection of NE increases D2 activity and mRNA within 1–2 h (206, 246). The mediators of the SNS response are the α- and β_{1-3}-adrenergic receptors, which act in a synergistic fashion. The α_1 receptors are implicated in this response because the administration of prazosin to intact rats blocks the cold-induced D2 stimulation in BAT (206). Studies in intact rats and isolated brown adipocytes confirm that NE induction of D2 depends on both α- and β-pathways and that these two pathways interact in synergistic fashion (247, 248, 433). cAMP is the logical common mediator of the cross-talk between α_1- and β-adrenergic pathways. As mentioned in *Section III*, the human, rat, and mouse *Dio2* genes contain a highly functional, canonical CRE binding protein binding site in the promoter (Fig. 12 and Ref. 195).

Blockade of D2-catalyzed T_4-to-T_3 conversion by iopanoic acid blocks the thermogenic response in T_4-treated hypothyroid rats, confirming the essential role of this enzyme (421). Because SNS-mediated D2 stimulation rapidly saturates the BAT T_3 receptors (Fig. 22), the physiological changes that take place during cold exposure in BAT reflect a composite interaction between NE- and T_3-generated signals that eventually lead to sustained heat liberation. This local D2-mediated hyperthyroidism requires T_4 in that the cold-induced increase in total body O_2 consumption is significantly greater in T_4-replaced than in T_3-replaced thyroidectomized rats.

Moderate systemic hypothyroidism does not prevent cold-induced saturation of the TRs (Fig. 22 and Ref. 423). In fact, BAT D2 is increased during hypothyroidism, in part by a reduction of T_4-induced D2 proteolysis (see *Section III*). In addition, hypothyroid rats have increased BAT-sympathetic activity in response to the reduced BMR, due to hypothalamic activation of local adaptive thermogenesis to sustain core temperature at 21–22 C. The accompanying increase in D2 activity compensates for the fall in circulating T_4 because treatment with T_4 at only 25% of the daily replacement dose increases BAT TR occupancy to approximately 50%, almost exclusively due to locally generated T_3 (423). Thus, BAT D2 functions as a strategic modulator of the thyroid impact in

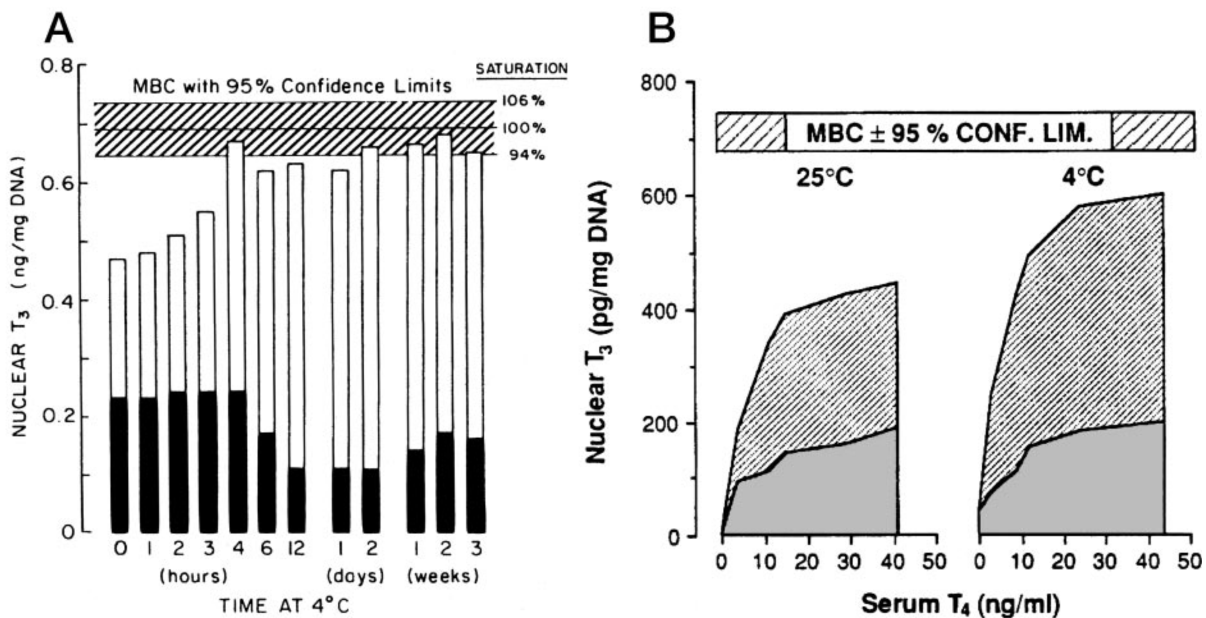

FIG. 22. Sources of T_3 and fraction of TRs occupied in brown adipose tissue during cold exposure in normal rats (A) or T_4-treated hypothyroid rats (B). In A, the *black portion of the bars* represent T_3 derived from plasma, $T_3(T_3)$, and *the open portion* represents T_3 derived from local D2-catalyzed T_4 5′ deiodination, $T_3(T_4)$. The *horizontal hatched zone* is the maximum binding capacity (MBC) of the TRs. In B, the animals were thyroidectomized and received different doses of T_4, resulting in various levels of serum T_4. In each *curve*, the *upper hatched zone* is $T_3(T_4)$ and the *lower gray zone* is $T_3(T_3)$. [Reprinted with permission from A. C. Bianco *et al.*: *Am J Physiol* 255:E496–E503, 1988 (434); and S. D. Carvalho *et al.*: *Endocrinology* 128:2149–2159, 1991 (423). © The Endocrine Society.]

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the brown adipocyte. SNS stimulation of D2 results in BAT hyperthyroidism (434). Furthermore, increases in D2 minimize the impact of the hypothyroxinemia observed during iodine deficiency and hypothyroidism. This is also true during fetal life, when BAT thermogenesis is not activated but BAT D2 activity is typically much higher and T_3 probably plays a role in BAT development (435). Maternal thyroidectomy does not change fetal BAT thyroid hormone concentration, as BAT D2 activity increases by 30–50% (436).

An additional role played by D2 and thyroid hormones in BAT is to mediate the 3- to 4-fold increase in the activity of lipogenic enzymes, *i.e.*, malic enzyme and glucose 6-phosphate dehydrogenase, observed in this tissue during cold exposure, a response that is also blunted in hypothyroid rats (420, 437). T_3, in turn, stimulates these enzymes (420, 437), including the expression of Spot-14, a lipogenesis-related protein, in differentiating brown adipocytes (438, 439). However, lipogenesis is paradoxically increased in the BAT of hypothyroid rats (440). The reduction in BMR and obligatory thermogenesis is accompanied by a compensatory increase in BAT-sympathetic activity (441), which combined with increased D2 activity, stimulates lipogenesis. During cold exposure, BAT lipogenesis is a very active pathway, accounting for more than 50% of the *de novo* fatty acid synthesis in the rat (442). BAT lipogenesis is particularly important because it generates the necessary fuel to sustain the high oxidation rate of BAT mitochondria. In freshly isolated brown adipocytes, NE stimulates lipogenesis (incorporation of tritiated water into lipids) and the activity of key lipogenic enzymes, *e.g.*, malic enzyme and acetyl-coenzyme A carboxylase, only in the presence of T_4- or TR-saturating concentrations of T_3. In their absence, NE markedly inhibits BAT lipogenesis and lipogenic enzymes. D2 blockade with iopanoic acid prevents the NE-mediated surge in lipogenesis in the presence of T_4, indicating its essential role in this process (443).

The 5- to 50-fold increase in BAT D2 activity during cold exposure suggests that this pathway might also serve as an extrathyroidal source of T_3 regulated directly by the hypothalamus and the SNS. This would explain the approximately 2-fold acute increase in plasma T_3 observed in small mammals during cold exposure, which in turn accelerates the metabolic rate and increases systemic thermogenesis (444). As an example, treatment of cold-exposed rats with PTU does not prevent the 10-fold increase in extrathyroidal T_3 production or the 6- to 8-fold induction of BAT D2, even though it inhibits more than 95% of D1 in liver and kidney. This indicates that this response is due to D2-catalyzed T_4-to-T_3 conversion. Similar results were detected in neonatal rats (445) and T_4-treated thyroidectomized rats, indicating that the TSH-stimulation of the thyroid gland plays a minor role in these acute physiological adaptations to cold exposure (446).

A similar situation is found in the newborn human, a transition period during which the fetus leaves the totally protected uterine environment. After birth, body temperature falls and the newborn responds by BAT-mediated adaptive thermogenesis, doubling the O_2 consumption within a few hours. This is initiated by a large catecholamine surge, which results in extremely high levels of NE and epinephrine in the cord blood. Plasma T_3 levels also rise markedly, approximately 3.8-fold in the first 90 min of extrauterine life (171). Although this is certainly in part due to the large TSH surge at delivery (447), given the widespread distribution and importance of BAT in neonatal thermogenesis, it is likely that BAT D2 contributes to this early plasma T_3 surge as well.

The regulation of BAT D2 by T_3 is unique. Despite the fact that T_3 decreases D2 mRNA in brain (215) and skeletal muscle (235), in BAT and cultured brown adipocytes T_3 potentiates the adrenergic stimulation of D2 (20-fold) by a mechanism that requires *de novo* protein synthesis (327, 328). This T_3 effect seems not to be D2-specific, as the overall adrenergic responsiveness of brown adipocytes is increased by treatment with T_3, amplifying the induction of several BAT cAMP-dependent genes, of which UCP1 is the typical example.

As discussed above, the source of our understanding on how thyroid hormones and D2 interact with the SNS to modulate BAT function and adaptive thermogenesis comes from studies that have been performed in hypothyroid animals. Most of these focused on the role played by D2 in mitigating the effects of hypothyroxinemia on brown adipocytes' function and UCP1 expression after T_4 administration. Evidence for the direct involvement of D2 in adaptive thermogenesis in intact animals has recently been provided by studies of mice with a targeted disruption of the *Dio2* gene (see *Section VII.B*).

F. Summary

The above discussion illustrates the diverse biological functions played by the deiodinases in the whole organism and in the tissue-specific regulation of T_3 concentrations. All this can occur in the absence of changes in T_4 secretion even though feedback regulation at the hypothalamic-pituitary level is one of the most thoroughly documented examples of the essential role for D2 in thyroid homeostasis (9, 331). The intricate interrelationships among the deiodinases, their actions in peripheral tissues, and their role in monitoring both the prohormone T_4 and active hormone T_3 are summarized in Fig. 23. The complexity of these interconnecting pathways illustrates the capacity for the sophisticated local regulation of thyroid status, which is dependent on the existence of the selenodeiodinases.

VI. The Deiodinases in Human Pathophysiology

A. Alterations in iodothyronine deiodination in the response to fasting or illness

It has been recognized for decades that there are significant changes in the concentrations of circulating thyroid hormones during illness or starvation in human plasma. Despite numerous studies, there remains much controversy regarding both the precise etiology of these changes and what, if anything, should be done therapeutically regarding them (448–450). The hallmark of these responses is a decrease in circulating free T_3 and an increase in total rT_3, although there is not complete agreement even on these most basic changes (177, 451, 452). The similarity of the changes in illness to those of fasting or caloric deprivation led to the concept that the

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decrease in thyroid hormone activation was a beneficial physiological response designed to reduce metabolic rate and to conserve protein during a period of stress (453). This has also been challenged (454). The issue of whether the reduction in serum T_3 may, in fact, be pathological and contribute to a worsening of the clinical status of the severely ill individual has been raised, although controlled studies have not shown beneficial effects of T_4 or T_3 supplementation in such individuals (455, 456). In patients' post coronary artery bypass grafting, however, there is disagreement about the effectiveness of T_3 supplementation, with one study showing a positive effect (457, 458).

The changes in circulating thyroid hormones and TSH during illness are a continuum with progressively greater abnormalities as the illness becomes more severe. Patients with mild illnesses, such as those that occur after uncomplicated surgery or during fasting, generally have a reduction of up to 50% in circulating T_3, a reciprocal increase in serum rT_3, and no changes in serum T_4 or TSH (Table 8 and Refs. 177 and 459). With moderately severe illness, the clearance of T_4 is slowed, whereas T_4 secretion persists, sometimes leading to an increase in free T_4 accompanied by further decreases in serum T_3 and increases in rT_3. In the most severely ill patients, TSH is suppressed, serum free T_4 falls, serum free T_3 becomes undetectable, and serum rT_3 increased, normal, or reduced depending on the concentration of free T_4. Patients with this degree of abnormality in thyroid function have a significantly increased mortality that is compatible with the concept that the most extreme abnormalities represent a deterioration in thyroid function secondary to a preagonal phase of severe illness (460, 461). Because abnormalities in iodothyronine deiodination occur as an early manifestation of illness as well as during caloric deprivation, we will focus on the basis for these rather than reviewing the entire spectrum of what has been termed the "euthyroid sick syndrome," "nonthyroid illness," or "the low T_3 syndrome." Such terms are the shorthand for the pattern of changes in thyroid function commonly seen in sick patients and reflect the endocrinologists' perspective about systemic illness.

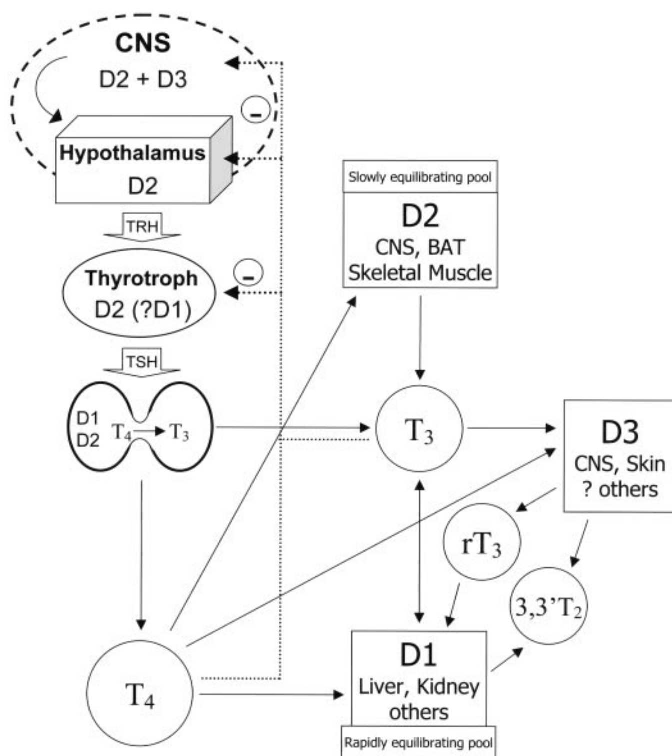

FIG. 23. Schematic diagram of the human thyroid axis depicting the role and probable tissue location of D1, D2, and D3 in the production and inactivation of plasma T_3 and in feedback regulation of thyroid function.

1. Etiology of changes in rT_3. When the abnormalities in T_3 and rT_3 were first described, the initial assumption was that these reciprocal changes reflected a diversion from T_4 activation to its inactivation. This raised the possibility that the changes could be attributed to an alteration in the specificity of D1 from 5′-to-5 T_4 deiodination because this is the only deiodinase with the capacity to catalyze both ORD and IRD of T_4 (Fig. 1). Subsequent studies indicated that the elevation in rT_3 was due to a reduction in the clearance of this T_4 byproduct, and its production rate is unchanged as long as T_4 remains normal (459, 462). This indicates that those tissues in which rT_3 is produced from T_4, largely by the action of D3, are deiodinating T_4 at least at normal rates during illness or fasting (Table 9). On the other hand, because the principal pathway for rT_3 clearance is via D1, these results indicate either that the D1 enzyme or its cofactor is reduced, or that the uptake of rT_3 into D1-expressing tissues is impaired (463). The latter is the likely explanation for this, given the minor impact of the 80–90% decrease in D1 activity in liver and kidney on serum rT_3 concentrations in the C3H mouse (see *Section VII.A*). Decreased transport of rT_3 into the D1-containing liver or kidney during fasting or illness has been attributed to either ATP depletion or interference with rT_3 transport by competing substances circulating in plasma (343, 448).

2. Etiology of the reduction in serum T_3. In moderate to severe illness, the serum T_3 can fall to 20–30% of baseline. As outlined in *Section V*, about 20% of T_3 in human plasma derives from the thyroid, with the T_3 derived from extrathyroidal D1- and D2-catalyzed T_4 5′ monodeiodination accounting for the remainder of the plasma T_3 (Fig. 17). Because TSH, and therefore T_3 secretion, is not suppressed unless illness is prolonged and/or severe, the severely reduced T_3 in most ill patients is primarily due to decreased peripheral T_4 deiodi-

TABLE 8. Modifications of thyroid-related hormones during fasting or illness

Severity of illness	Thyroid-related hormones			
	Free T_4	Free T_3	Total rT_3	TSH
Mild	Normal	Reduced up to 50%	Increased up to 2-fold	Normal
Moderate	Increased	Reduced up to 90%	Increased up to severalfold	Normal
Severe	Reduced	Almost undetectable	Variable	Reduced

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TABLE 9. Potential causes of the reduced extrathyroidal contribution of various sources of plasma T_3 in mild to moderate illness or during caloric deprivation in humans

T_3 production	Evidence for reduction due to		
	tissue T_4 uptake decrease	deiodinase protein decrease	cofactor deficiency
D1 catalyzed	Yes	No	Yes (in rats)
D2 catalyzed	Yes	Probable	?

nation by D1, D2, or both. The fact that the fall in T_3 substantially exceeds what we can reasonably assign to D1 (25%) suggests that plasma T_3 generation by D2 must also be inhibited. With respect to D1-containing tissues, T_4 uptake into the rapidly equilibrating pool, primarily liver and kidney, is significantly reduced in obese patients on a 240-kcal diet, and similar observations have been made in uremia (464). This can explain the decrease in T_3 production via D1, and again, either inhibition of T_4 transport by unknown circulating compounds or by ATP depletion could be to blame (343, 448). In addition, entry of T_4 into the slowly equilibrating pool, likely to be the one in which D2-catalyzed T_3 production occurs, is also reduced in obese patients on a hypocaloric diet (464). In addition to reduced T_4 transfer, a second important consideration with respect to D2-catalyzed T_4-to-T_3 conversion is the potential for the rapid proteolysis of the short-lived D2 through the ubiquitin-proteasome pathway. Thus, because persistent D2 synthesis is required to maintain D2 at normal levels that may not be maintained during fasting or illness. It is tempting to speculate that a rapid fall in D2 protein can explain the abrupt decrease in plasma T_3 associated with these conditions. The possibility that D3 action is also increased during illness must also be considered, but there are few data bearing on this possibility.

To the extent that T_3 production is reduced as an inevitable consequence of caloric stress or illness and that D2, unlike D1 or D3, is a protein with an extremely short half-life, it seems likely that a reduction in T_3 production due to D2 deficiency could be a programmed response to nutritional stress at least in humans. This would support the teleological explanation that the reduction in serum T_3 is beneficial, rather than pathophysiological, at least in mild to moderate illness. This would imply, in turn, that T_3 supplementation in these circumstances would not be physiologically appropriate even though we do not have clear documentation as to what advantages arise from the impaired T_3 production.

B. D3 overexpression in hemangiomas causes consumptive hypothyroidism

Until quite recently, discussions of the pathophysiological role of the deiodinases in various clinical states focused on decreases in D1 or D2 activity during illness or the consequences of a blockade of T_4-to-T_3 conversion by various agents such as amiodarone. A distinct exception to this is the recent discovery that high levels of D3 are present in infantile hemangiomas (276). If these tumors are sufficiently large, the rate of thyroid hormone inactivation can exceed the maximal rate of thyroid hormone synthesis in the infant. The first patient documented with this condition was 3 months old, presenting with severe hypothyroidism with an elevation in serum TSH, undetectable serum T_4 and T_3 concentrations, and high rT_3 and Tg (Fig. 24). To reverse the clinical hypothyroidism rapidly, iv therapy with liothyronine (T_3) and levothyroxine (T_4) was instituted with the rate of infusion titered to normalize TSH. It was thus possible to approximate the inactivation rate of T_3 and T_4 from the infusion rate, which was 96 μg of T_3 plus 40 μg of T_4/24 h. When the T_3 degradation rate is converted to the amount of orally administered levothyroxine, which would be required to generate it (assuming 33% T_4-to-T_3 conversion and 80% absorption), this amounts to 8–9 times the amounts of T_4 required for adequate replacement of an athyrotic infant of this age (37–50 μg levothyroxine per day). Remarkably, even during the infusion at such a high rate, serum T_3 concentrations barely reached the normal range and serum T_4 was never detectable. However, rT_3 rose rapidly to extremely high levels when T_4 was given, providing direct evidence of its IRD (Fig. 24). The possibility of previously unrecognized congenital hypothyroidism in this infant was unlikely because of the normal bone age, the enlarged thyroid in a normal location by magnetic resonance imaging, and a serum Tg of approximately 1000 ng/ml. The latter is markedly elevated over the upper limits of normal in this age group (465). D3 activity was subsequently identified in the hepatic hemangioma at levels 8 times that in human placenta (780 fmol/min·mg protein), and *in situ* hybridization localized the D3 mRNA to hemangioma cells. Retrospective review of patients with hemangiomas and hypothyroidism at The Children's Hospital in Boston identified two others with similar pathophysiology. Three of five samples of hemangioma tissue from either cutaneous or hepatic hemangiomas contained D3 activity in the range found in human term placenta, and other similar patients have now been reported (466). Thus, the pathophysiology of this newly recognized cause of "primary" hypothyroidism is inactivation of circulating T_4 and T_3 more rapidly than the normal thyroid can secrete it despite intensive stimulation by endogenous TSH. We believe that the term "consumptive hypothyroidism" is appropriate to describe this syndrome.

The relationship between infantile hemangiomas and D3 expression is especially significant because it identifies a cause of hypothyroidism that occurs at a critical age for neurological development. Although extensive hepatic hemangiomas can be fatal, a significant fraction of these infants survive with therapy and the natural propensity of these tumors to regress. Accordingly, these patients will usually require replacement with large quantities of thyroid hormone in addition to therapy directed at their hemangiomas. Thyroid hormone treatment is imperative to prevent the complication of irreversible mental retardation.

Hemangiomas produce high quantities of bFGF, which has been shown to activate the expression of D3 in rat glial cells via ERK activation (310). It seems possible that this is one mechanism for the high D3 expression in these tumors. Studies are underway to determine which specific biochemical events occur in hemangioma cells and to discover whether D3 overexpression can be ameliorated pharmacologically.

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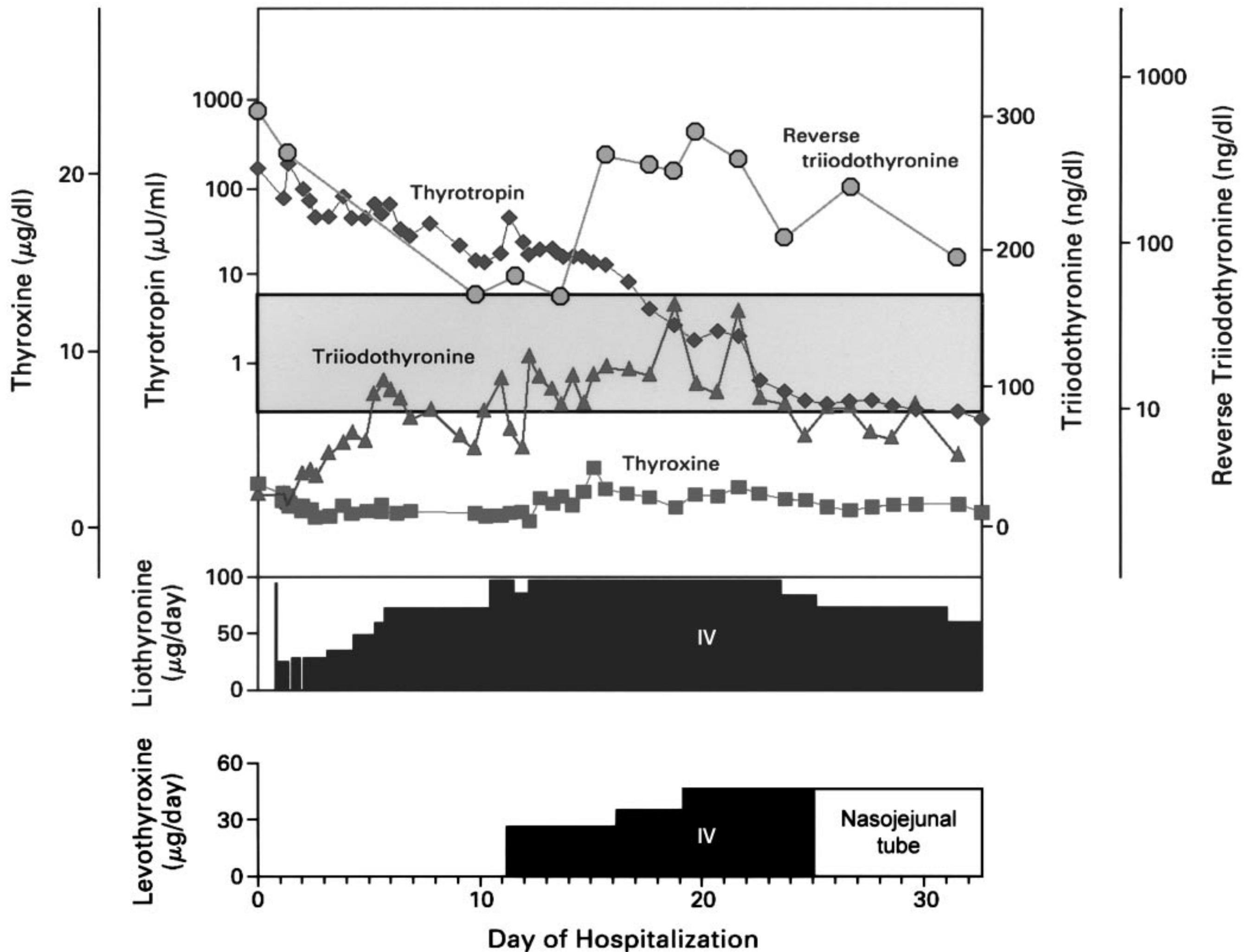

FIG. 24. Thyroid function and treatment during hospitalization in an infant with multiple hepatic hemangiomas. The *shaded region* is the normal range for serum TSH concentrations (0.3–6.2 μU/ml; *diamonds*), serum T_4 concentrations (6.8–13.3 μg/dl; *squares*), serum T_3 concentrations (86–170 ng/dl; *triangles*), and serum rT_3 (10–50 ng/dl; *circles*). Each value is plotted related to the normal range. Serum TSH and rT_3 concentrations are plotted on a logarithmic (base 10) scale. The infant was treated with iv infusions of both liothyronine and levothyroxine. The route of administration of levothyroxine was changed to nasojejunal on d 25 of hospitalization. [Reprinted with permission from S. A. Huang *et al.*: *N Engl J Med* 343:185–189, 2000 (276). © Massachusetts Medical Society.]

C. D1 overexpression contributes to the relative excess of T_3 production in hyperthyroidism

The production rate of T_3 and its circulating concentration is about 2-fold higher relative to that of T_4 in hyperthyroid patients (127). This is reflected in the markedly greater elevation in free T_3 than in free T_4 in such patients. Because the human *Dio1* promoter is T_3 responsive (Fig. 10), one would anticipate that D1 activity or mRNA would be significantly increased in hyperthyroid patients. This has been demonstrated in Graves' thyroid tissue and in circulating mononuclear leukocytes in patients with Graves' disease (84, 467, 468). It would be expected that PTU, a drug that blocks D1 but not D2 activity, would have a greater effect on plasma T_3 production in thyrotoxic than euthyroid individuals because D1 activity should be increased and D2 activity reduced in such patients. Such studies require taking into account any acute effects of PTU on thyroidal T_3 synthesis by comparing it with the effects of methimazole. Furthermore, it is necessary to reduce T_3 secretion by the Graves' thyroid to as great an extent as possible with iodide to allow a focus on peripheral T_3 production. That PTU causes a marked inhibition of T_4-to-T_3 conversion was demonstrated by comparing the acute changes in serum T_3 between Graves' patients treated with a combination of iodide and PTU with those in a similar group treated with methimazole and iodide (127). PTU plus iodide caused an approximately 50% greater fall in the T_3/T_4 ratio in plasma after d 1 than did iodide and methimazole. A dose-response relationship of the decrease in the T_3 at 24 h and the dose of PTU was evident with doses up to 1600 mg of PTU/d (Fig. 25). PTU at doses of 1 g/d or higher decreased T_3 over 50% in 24 h. No patient receiving any dose of methimazole plus iodide had more than a 30% decrease in plasma T_3 at 24 h. These results indicate that a PTU-inhibitable process, D1-catalyzed T_4-to-T_3 conversion, is more active in the hyperthyroid than the euthyroid subject in whom PTU causes 0–25% decrease in T_3 (136, 333, 334). This has led

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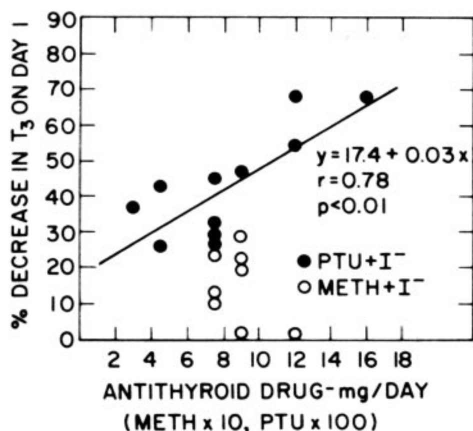

FIG. 25. Acute effects on serum T_3 of treatment of patients with hyperthyroidism due to Graves' disease with PTU plus iodide (I^-) *vs.* methimazole (METH) plus iodide for 24 h. There is a significant correlation between the percentage of decrease in T_3 at 24 h and the dose of PTU up to 1600 mg/d, but not with that of methimazole. [Data from: J. Abuid and P. R. Larsen: *J Clin Invest* 54:201–208,1974 (127).]

to the recommendation that large doses of PTU or other agents that block T_4-to-T_3 conversion, such as iopanoic or ipodipic acid, be used in the acute treatment of the severely hyperthyroid individual (469–471). This is supported by the results of a crossover comparison study showing that the more rapid decrease in T_3 with PTU than with methimazole is mirrored in a more rapid clinical response (472).

A paradoxical observation in the Graves' thyroid is that thyroidal D2 mRNA is increased despite systemic thyrotoxicosis (Table 5 and Ref. 201). This is presumably due to the effect of the thyroid immunostimulator to activate the cAMP-dependent human *Dio2* promoter, which must overwhelm the negative transcriptional effect of T_3 on human *Dio2*. In some patients receiving antithyroid drugs, the presence of D2 activity in the Graves' or TSH-stimulated human thyroid raises the possibility that some of the excess T_3 secretion could result from intrathyroidal T_4-to-T_3 conversion catalyzed by D2 (201).

D. Effects of inhibition of deiodinase function during therapy with amiodarone

Amiodarone is a potent iodine-containing cardiac antiarrhythmic that shares some structural homology with thyroid hormones. Thyroid function tests are usually abnormal during its administration as a result of the compensation in response to the effects of this drug on various aspects of thyroid physiology (473, 474). Amiodarone affects iodine supply because it is 37% iodine by weight. A maintenance dose of approximately 300 mg/d increases iodine intake about 1000-fold, dramatically expanding the iodine pool. Consequently, significant thyroid dysfunction may develop in some patients during long-term treatment (475–477). In addition, amiodarone or one of its metabolic products may also interfere with T_3-TR interaction (475). In the present discussion, only the effects of amiodarone on the metabolism of thyroid hormones will be addressed.

Almost all patients receiving amiodarone will develop an initial decrease in circulating T_3 and an increase in rT_3 secondary to the effects of amiodarone (or one of its metabolic byproducts) to inhibit D1 and perhaps D2. As a consequence, T_4 and TSH secretion increase raising the free T_4 concentration and normalizing that of T_3 (475, 476). Similar changes are also seen in hypothyroid patients receiving levothyroxine replacement therapy. The dose of levothyroxine must be increased to compensate for this (478).

Thyroid hormone kinetics are predictably affected by amiodarone. In humans, the effects on T_4 production rate depend on the duration of treatment and vary from a slight (10%) decrease after 3 wk (479) to no change (5–6 wk; Ref. 480) and approximately 100% increase after 9 months (479). T_4 and rT_3 MCR are reduced 20–25% in all studies, and the plasma T_4 and rT_3 half-lives are prolonged accordingly. There is a reduction in the T_4-to-T_3 conversion rate from 26 to 43% to 10 to 17% during amiodarone therapy, but because T_4 production is increased, net T_3 production and plasma free T_3 concentrations normalize (479–481).

Studies in animals allow a better understanding of the biochemical changes in thyroid hormone metabolism induced by this agent. In rats given amiodarone, D1 mRNA levels are normal (482), but the enzyme activity is inhibited in homogenates of liver, heart, and kidney in a dose-dependent fashion (483–487). The same is observed in hepatocytes exposed to amiodarone (488). In rats acutely treated with amiodarone, pituitary 5′ deiodination (largely by D1) falls 60–70% (489). To our knowledge, no study has specifically quantified D2 activity in animals treated with this agent. In two studies, however, the effect of amiodarone on D2 activity was tested *in vitro*. In pituitary homogenates, the addition of amiodarone inhibited only 13% of D2 activity, an effect that was not dose dependent (490), and in human skin homogenates the inhibition was 33% (222). However, the limited solubility of the drug makes it difficult to examine the effect of concentrations higher than 10^{-4} M, which can be present in liver and adipose tissue in patients receiving this drug (491). The mechanism of inhibition of D1 in amiodarone-treated animals is likely to be competitive inhibition with substrate (492).

In addition to an inhibitory effect on deiodination, amiodarone inhibits the active transport of T_4 and T_3 into hepatocytes and pituitary cells (493, 494). The resulting decrease in availability of T_4 and rT_3 will decrease the net production of T_3 and 3,3′-T_2, respectively. The amiodarone-induced transient increase in plasma TSH occurs in response to the early decrease in plasma T_3 as well as to possible effects of the drug to inhibit T_3 binding to pituitary TR (475, 476, 493). In addition, if D2 activity is inhibited by amiodarone, then a fall in the TR saturation in the thyrotroph would further increase TSH synthesis and secretion, leading to the subsequent increase in T_4 production.

Taken together, the effects of amiodarone are quite similar to those observed during administration of one of the iodoaniline gall bladder-visualizing agents such as iopanoic acid (470, 495). These are characterized by increases in T_4 and rT_3 and decreases in T_3, which would be expected from the competitive inhibition of D1 and D2 by this and similar agents, as discussed elsewhere (319, 496). There is as yet no evidence of a relationship between the effects of amiodarone on thyroid hormone metabolism and its therapeutic effects,

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although the recognition that D2 mRNA is expressed in human myocardium raises that issue (17, 201).

VII. Effects of Genetic Alterations in Deiodinase Expression

A. Effects of a spontaneous genetic deficiency in Dio1 gene expression

A mouse with a targeted *Dio1* gene inactivation has not yet been reported. One reason for this is that a polymorphism in the mouse *Dio1* gene anticipates the effects of a *Dio1* knockout on thyroid physiology. This polymorphism was first discovered by analyses of the deiodination of radioiodinated 2,3,7,8-tetrachlorodibenzo-*P*-dioxin in a number of different mouse strains (69, 497). The ^{125}I-substituted 2,3,7,8-tetrachlorodibenzo-*P*-dioxin derivative is a substrate for D1, and its metabolic clearance occurs at different rates in different mouse strains, with most, such as the C57/BL6 (C57) strain, showing a relatively high deiodinative clearance. However, clearance of iododioxin in the C3H/HeJ (C3H) strain was about one-tenth that in most other species. The difference between the two extreme examples was explained by a 10- and 5-fold lower expression of the D1 in liver and kidney as measured by activity and BrAcT_3 labeling, paralleled by lower D1 mRNA content in these organs, respectively (69, 497). Restriction fragment analyses and mapping indicated that there was a restriction fragment polymorphism difference segregating with low expression of *Dio1* that could be seen on a *Taq*I genomic digestion. Crossover genetic mapping localized *Dio1* to mouse chromosome 4 about 3 centimorgans proximal to the *GLUT-1* gene (69).

The promoters of the C57 and C3H *Dio1* genes differ with a 21-bp insert in the C3H *Dio1* gene containing five CTG repeats starting at position −371 (70). The 5′-FR containing this portion of the gene directed 2- to 3-fold less transient CAT expression than did the C57 promoter. This may contribute to the lower mRNA expression in the C3H and other strains containing this haplotype (70). The restriction fragment polymorphism difference between the high- and low-expressing haplotypes is due to a 150-bp expansion in intron 2 of the C3H gene. This cosegregates with the CTG repeats but does not affect *Dio1* expression (70).

The C3H mouse provides a genetic model in which one may evaluate the effects of a substantial, life-long decrease in D1 expression in an intact healthy vertebrate. It is at first surprising that 90% and 75% reductions in D1 activity in liver and kidney, respectively, do not affect serum T_3 concentrations although there is an increase in rT_3 (Table 10 and Refs. 69, 128, and 497). A partial explanation for the normal T_3 is found in the increased plasma free T_4 concentrations in the C3H mouse (69). Thus, the ratio of free T_3 to T_4 in the C3H mouse is approximately half that in the C57 strain. This indicates a significant role for D1 in the generation of plasma T_3 in the mouse, as also occurs in the rat (see *Section V.B.1.b*). The decrease in D1 expression also modestly reduces the metabolic clearance of T_3 (128). C3H mice given the same dose of parenteral T_3 have roughly 2-fold higher circulating T_3 concentrations 24 and 48 h later than do C57 mice of the same weight (128). This is in agreement with the modest impairment of T_3 clearance in PTU-treated rats (168). Thus, D1 deficiency may have a greater effect on peripheral T_4-to-T_3 conversion than suggested by the 2-fold decrease in the ratio of free T_3 to T_4 in the circulation. The chronic hyperthyroxinemia of the C3H mouse reduces the D2 content in the CNS and pituitary presumably due to accelerated D2 ubiquitination caused by the higher plasma free T_4 (69, 188). Although basal TSH could not be accurately quantified due to artifactual effects of mouse serum in the TSH assay, the TRH-induced TSH release is normal in the C3H mouse, indicating that the hypothalamic-pituitary-thyroid axis is not affected by the impaired D1-catalyzed T_4-to-T_3 conversion (69). Although no studies of the sources of T_3 and of the relative contributions of $T_3(T_3)$ and $T_3(T_4)$ to nuclear TR in the brain and pituitary of C3H animals have been reported, one would predict that these would be similar to those in mice with normal D1 activity, due to a combination of the suppressed D2 activity and increased circulating T_4.

TABLE 10. Physiological analysis of the effects of a genetic decrease in *Dio1* or *Dio2* gene expression

Strain	Plasma				Activity in various tissues		
	TSH	T_4	T_3	rT_3	D1	D2	D3
C3H/HeJ	=	↑	=	↑	↓↓↓*	↓	=
D2 gene disruption	↑	↑	=	?	=	No activity	↑↑

Figures based on results in Refs. 24, 69, 70, 128, 497; *, liver and kidney estimates.

These results suggest that the phenotype of a genetically D1-deficient human would be an increased serum T_4 with normal serum T_3 and TSH. One such family has been described with a putative generalized 5′ deiodinase defect (498). There is no abnormality in the *Dio1* coding sequence or promoter and 5′-FR up to 2.5 kb in the propositus (499). The only difference was an exchange of G for A in the 5′ half-site of TRE2 (GGGTCA *vs.* AGGTCA; see Fig. 10). This change caused no difference in the response of this promoter to T_3. It is of interest, however, that a similar phenotype has been observed in a patient who has a defect in the transport of T_4 into the liver (500). A decrease in T_4 transport could also explain the abnormality in the Israeli family (498).

B. Effects of targeted disruption on the Dio2 gene

Mice with targeted disruption of the *Dio2* gene were recently developed in a C57BL6/129SV-strain background by replacement of the sequences encoding amino acids 74–266 and part of the 3′ UTR (nt 2769 in GenBank accession no. MN 010050) with a neomycin resistance cassette (24). These animals have no D2 activity in the pituitary, cerebral cortex, or BAT even under hypothyroid conditions. This leads to the expected impairment of feedback regulation in the intact mouse with serum T_4 concentrations increased about 2-fold over those in wild-type mice (Table 10). The serum T_3 concentrations in $Dio2^{-/-}$ mice are normal. Thus, this situation resembles that found in the endogenous *Dio1*-deficient C3H mouse, in which precisely similar perturbations have occurred in the circulating T_4 and T_3 concentrations. The increase in serum T_4 can be explained by both central and peripheral consequences of D2 deficiency. The absence of D2 will make T_4 unable to exert feedback regulation at either the

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hypothalamic or pituitary level. It seems unlikely that the concentrations of D1 in mouse pituitary are sufficient to generate significant quantities of T_3 given the inefficiency of that enzyme in T_4 ORD (201). It is not known to what extent D2-catalyzed T_4-to-T_3 conversion contributes to plasma T_3 in the mouse. Based on studies in the rat, about 50% of peripheral T_3 production would be expected from D2 and another 50% from D1-catalyzed T_4-to-T_3 conversion (see *Section V.B*). Thus, an increase in serum T_4 must occur to compensate for the absent contribution of the low-K_m D2 to the peripheral T_4-to-T_3 conversion process. However, there is also a persistent 2-fold increase in the circulating TSH concentration. This will lead to increased thyroidal T_4 and T_3 secretion, another mechanism by which these animals compensate for the absence of D2. The fact that the animals survive cold exposure (see below) and appear to be at least grossly normal in their growth and development exemplifies the redundancy of the systems for thyroid hormone activation in the intact animal. To be sure, it will be of great interest to explore the neurodevelopmental consequences of the absence of D2 in the CNS, given its major role in the process by which T_3 enters the nuclei of these cells (190). One would certainly expect difficulties in cochlear development, and these have been verified in a recent abstract (501).

D2 is well recognized as an important enzyme in BAT physiology based largely on studies of hypothyroid rats (see *Section V.E*). These have focused on its role in mitigating the effects of hypothyroxinemia on brown adipocytes. Although this is relevant for understanding the role of D2 in sustaining adaptive thermogenesis during iodine deficiency (355), there is little information defining the role of D2 in this process in iodine-sufficient animals (425, 502). Recent studies using the *Dio2*$^{-/-}$ mouse provide the first direct evidence that D2 is also required for the normal response to cold stress in a normal mammal. Despite a normal plasma T_3 concentration, cold-exposed *Dio2*$^{-/-}$ mice become hypothermic due to impaired BAT thermogenesis and survive by compensatory shivering with consequent acute weight loss. This occurs despite normal basal mitochondrial UCP1 concentration. In *Dio2*$^{-/-}$-isolated brown adipocytes, the acute NE-, CL316,234 (a β3-selective agonist)-, or forskolin-induced increases in lipolysis, UCP1 mRNA, and O_2 consumption are all reduced due to impaired cAMP generation. These hypothyroid-like abnormalities are completely reversed by a single injection of T_3 14 h earlier. Thus, the SNS-responsive *Dio2* gene in BAT is essential to support its basal adrenergic responsiveness as well as the development of the intracellular thyrotoxicosis, which permits thermal homeostasis of small mammals with a minimum of caloric expenditure (503).

C. Isolated myocardial D2 overexpression causes cardiac thyrotoxicosis

The heart is one of the most sensitive organs to variations in plasma thyroid hormone level. Thyroid hormone can increase myocardial inotropy and heart rate as well as dilate peripheral arteries to reduce afterload. At a molecular level, many cardiac genes respond to thyroid hormone: among them, the myosin heavy chains (MHC), the hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), and the sarcoplasmic reticulum calcium ATPase. The expression of D2 in human cardiac and skeletal muscle and its absence from the corresponding tissue in rodents is one of the most intriguing differences in mammalian deiodinase physiology. It raises the possibility that, in humans, this tissue can respond not only to changes in plasma T_3, but also to those in T_4, thus resembling the pituitary and brain. Furthermore, the presence of an intracellular T_3-producing enzyme in the human heart may preserve this organ from the systemic reduction in thyroid hormone concentrations in iodine deficiency, although the potential contribution of cardiac D2 to intracellular or peripheral T_3 production is still unknown. However, it is clear that, based on the tissue distribution of D2 expression, rodents are not a faithful model of the human situation.

To provide a model that might better reflect the human myocardium with respect to sources of T_3, a transgenic mouse model has been prepared in which human D2 is highly expressed in the heart. This was obtained by inserting the human D2 coding sequence 3′ to the cardiac-specific mouse α-MHC promoter (504). These transgenic mice expressed high myocardial D2 activity, but surprisingly, the myocardial T_3 concentration was only minimally increased. The reason for this is under investigation. It is not due to rapid diffusion of T_3 from the myocardium because circulating T_3 and T_4 concentrations are normal. It could be due to low T_4 uptake by the mouse myocardium or to the lack of an endogenous cofactor for D2. Although plasma T_4 and T_3, growth rate, and heart weight were not affected by D2 expression, myocardial thyrotoxicity was detected in the performance of isolated hearts. Consistent with the effect observed with endogenous thyroid hormone-induced thyrotoxicosis, there was an increase of about 20% in heart rate and an 30% increase in the rate pressure product, *i.e.*, 284 $\pm$ 12 to 350 $\pm$ 7 beats/min. This was accompanied by an increase in pacemaker channel HCN2 but not in α-MHC or sarcoplasmic ER calcium ATPase (SERCA II) mRNA levels. The HCN2 gene is T_3 responsive in rats (505). Biochemical studies and ^{31}P nuclear magnetic resonance analysis demonstrated a significant reduction in phosphocreatine and creatine in transgenic animals, which may make the cardiac tissue of these mice more susceptible to ischemic challenge because hypoxia would cause greater myocardial depletion in ATP concentrations than occurs in the wild type.

These minimal changes were somewhat unexpected, because many of the alterations caused by short-term high-dose exogenous thyroid hormone did not occur. In humans, the clinical syndrome of modest increases in serum T_3 and T_4 (within the normal range) accompanied by suppressed TSH creates the state of "subclinical" hyperthyroidism. This can occur spontaneously, but it is intentionally induced by excess replacement with exogenous T_4 in patients with thyroid cancer. Because the changes demonstrated in these mice occur with minimal increases in myocardial T_3, these modest increases in circulating T_4 and T_3 are likely to have similar effects on the human myocardium. Supporting this concept, a recent report demonstrated an increase in heart rate in patients with normal thyroid hormone levels but suppressed TSH compared with age-matched controls (506). These results suggest that even mild chronic myocardial thyrotoxi-

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cosis can cause tachycardia and associated changes in high-energy phosphate compounds. This "humanized mouse heart" model is being evaluated to identify the consequences of mild chronic cardiac-specific thyrotoxicosis on cardiac function and to elucidate the direct effects of T_3 on the heart uninfluenced by effects of thyroid hormone-induced changes in total body metabolism.

VIII. Conclusions and Future Directions

It is clear that the selenodeiodinases play pivotal roles in thyroid physiology. Their conservation as selenoproteins throughout the vertebrate kingdom illustrates the biochemical advantages of Se- as opposed to S-reductive deiodination reactions and implies their physiological necessity. This conservation comes at a high price because synthesis of these proteins requires an entirely independent complex of gene products that differ from those required for the synthesis of virtually all other eukaryotic proteins (Fig. 4). We are only now going to be able to define the specific role of each of these enzymes with the application of gene targeting techniques.

In the final analysis, what are the advantages to the organism served by such an intricate system of activation and inactivation steps? T_4 formation is a complex process requiring the synthesis of a 660-kDa homodimer, Tg, to generate only 3–4 residues of this 777-Da molecule. This iodothyronine is virtually insoluble in water and circulates bound to one or more species-specific plasma proteins. T_4 dissociates from these, and small quantities of free T_4 are transported into cells in which they bind to cytosolic proteins. Up to this point, T_4 has no physiological effect because it does not enter the nucleus at high enough concentrations to occupy the ligand binding site of the DNA-bound TRα and β receptors. Thus, in physiological terms, it is a prohormone. Loss of a single iodine from the outer ring produces the active hormone T_3, which may either exit the cell (in D1-containing cells), enter the nucleus directly (in D2-containing cells), or possibly even both (*e.g.*, in human skeletal muscle). Whether the cytosolic T_3 originates from the plasma, $T_3(T_3)$, or is derived from T_4 within the cell, $T_3(T_4)$ (Fig. 23), when it enters the nucleus it has a high likelihood of binding to the TRs. This binding induces a molecular rearrangement of the TR resulting in the internal folding of helix 12 to cap the ligand-binding pocket (507, 508). The subsequent ligand-dependent rearrangements of the TR result in the dissociation of corepressors and the binding of coactivators to the RXR/TR complex or TR/TR, causing a switch from unliganded TR-mediated gene repression to liganded TR-mediated gene activation. Because neither T_4, rT_3, nor 3,3′-T_2 (Fig. 1) can serve this function at their respective physiological concentrations, it is the selenodeiodinases that initiate and maintain thyroid hormone effects.

Tissue-specific regulation of T_4 activation and T_4 and T_3 inactivation permits day-to-day, or even, in the hypothalamic-pituitary axis and perhaps other cells as well, minute-to minute variations in thyroid status (509). This can accomplish a myriad of biological goals that are especially critical in vertebrate development, as we have discussed. For example, during metamorphosis, T_3 can be inactivated in a specific region of the retina by D3, whereas D2 activates T_4 in tail tissues. In mouse brain, T_4 can be activated in the cochlea, whereas T_3 remains low in the remainder of the CNS. The control of thyroid hormone activation may also be general, such as occurs during human illness or fasting. It is impossible, either systemically or locally, to acutely reduce the plasma concentration of T_4 by stopping its production because it has such a long plasma half-life. However, a reduction of T_4 activation by D1 and D2, and/or even increased T_3 inactivation by D3, rapidly alters the thyroid status of the cell. Although we are still not certain as to the specific purpose served by this rapid decrease in T_3, the fact that cessation of thyroid hormone action is so uniformly a part of the response to fasting or illness in humans, however it occurs, suggests that there are intrinsic biological advantages which accrue because of it.

Although much has been learned about these enzymes in the last decade, there are huge gaps in our understanding of several fundamental issues, especially of human deiodinase physiology. Although we have concluded from our review that a significant fraction of plasma T_3 must derive from D2-catalyzed T_4-to-T_3 conversion, in large part this is by inference from studies showing that neither the thyroid gland nor the PTU-sensitive D1-catalyzed deiodination process seems to account for more than 40–50% of the plasma T_3. The fact that fractional T_4-to-T_3 conversion is increased in the hypothyroid or hypothyroxinemic individual is confirmation that a D2-regulated process occurs in humans, but this requires further direct testing. Moreover, whereas we know that the inner and outer rings of T_4 are deiodinated at roughly equal rates and believe the latter occurs largely via D3, we have not identified an anatomical location for this enzyme outside the CNS, except during pregnancy. Relatively speaking, if we are to judge from the quantities of D1 present in the liver and kidney and of D2 in skeletal muscle, one might speculate that there must be other cells that have low levels of D3 but are widely distributed. Perhaps, however, despite the absence of an effect of illness or of PTU on rT_3 production, D1 is a significant contributor to T_4 and T_3 inactivation. Again, the critical studies to resolve this issue have not been performed.

We hope this review will serve the purpose not only of summarizing the state of the art in this field but also of illuminating gaps in our knowledge such as those mentioned. We anticipate that some of our conclusions will be considered provocative. We also recognize, and apologize for, our brief discussions of a large body of early work in this field, which were dictated by space constraints. Finally, we have endeavored to communicate the sense of excitement that those of us working in this field are experiencing as we can now, after so many years of working only with deiodinase activity, take advantage of the exciting new techniques of molecular biology to learn more about how the intricate program of thyroid hormone activation and inactivation common to all vertebrates is regulated.

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