

## Thyroid hormone binding motifs and iodination pattern of thyroglobulin

Salvatore Benvenga<sup>1,2,3</sup>, Fabrizio Guarneri<sup>4</sup>

<sup>1</sup>Department of Clinical and Experimental Medicine - Endocrinology, University of Messina, via Consolare Valeria, Gazzi, 98125 Messina, Italy, <sup>2</sup>Master Program on Childhood, Adolescent and Women's Endocrine Health, University of Messina, via Consolare Valeria, Gazzi, 98125 Messina, Italy, <sup>3</sup>Interdepartmental Program on Molecular and Clinical Endocrinology and Women's Endocrine Health, AOU Policlinico G. Martino, via Consolare Valeria, Gazzi, 98125 Messina, Italy, <sup>4</sup>Department of Clinical and Experimental Medicine, Dermatology, University of Messina, via Consolare Valeria - Gazzi, 98125 Messina, Italy

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### 1. ABSTRACT

A phylogenetically conserved 5-residue thyroid hormone (TH)- binding motif was originally found in a few TH plasma carriers and, more recently, in all known plasma and cell-associated proteins interacting with TH as well as in proteins involved in iodide uptake. Minor variations of the motif were found, depending on the particular class of those proteins. Since thyroglobulin (Tg) is the protein matrix

for TH synthesis starting from iodination of a selected number of tyrosines (to form first monoiodotyrosine (MIT) and diiodotyrosine (DIT) and then T3 and T4), we hypothesized that by searching the presence of perfect or imperfect versions of that motif in two Tg species (human and murine) in which the iodinated tyrosines and pattern of iodotyrosine/iodothyronine formation are known, we could have found relevant

explanations. Explanations, which are not furnished by the simple possession of tyrosine-iodination motifs and sequence of the iodination motif, concern why only some (but not other) tyrosine residues in one species are iodinated and why they have a particular iodination pattern. In this bioinformatics study, we provide such explanations.

## 2. INTRODUCTION

In 1994, Benavenga *et al.* (1) found, using a bioinformatics approach, that the amino acid sequences of apolipoproteins known as minor plasma carriers of thyroid hormone (namely, apo A-I, A-II, A-IV, C-I, C-II, C-III, apo B-100, and apoE) have local homology with those of major plasma carriers (namely, thyroxine-binding globulin (TBG), prealbumin or transthyretin (TTR) and serum albumin (HSA)). In a subsequent paper (2), the conservation of the 5-residue hydrophobic TH binding motif “Y, L/I/M, X, X, V/I/L” (where X indicates any amino acid) was demonstrated in all known apolipoproteins. In 2016, using more advanced technologies and a much larger sequence database, it was shown that the 5-residue motif is conserved in human and animal TH binding proteins and absent in those which do not bind TH; furthermore, it was shown that the 5-residue motif is part of a larger motif (3).

More recently, we tested the hypothesis that the motif was possessed by another category of cell proteins that interact with TH, the selenodeiodinases (4). The relationship of selenodeiodinases (dio-1, dio-2 and dio-3) with TH is one of the enzyme-substrate kind, in that they activate the prohormone T4 into the more potent T3 and/or inactivate either TH into poorly active metabolites. Interestingly, upon analyzing a total of 488 sequences of selenodeiodinases from *Homo sapiens* and another 487 species, we noted that the 5-residue motif was conserved imperfectly. Precisely, the consensus sequence was F, L/V/M, L/I/V, V/I, Y, with the notable presence of Tyr in the fifth position in lieu of any residue of the amino acid group Ile/Leu/Met/Val.

We realized that an additional protein, thyroglobulin (Tg), interacts physically with TH, in that TH synthesis takes place starting from iodination of certain (not all) tyrosine residues of Tg to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). Subsequent coupling of these two iodotyrosines leads to formation of triiodothyronine (T3), while coupling of two DIT molecules leads to formation of tetraiodothyronine (T4). On the other hand, it is long known that iodotyrosines can be bound by plasma proteins, with an affinity of approximately  $1 \times 10^{-6}$  M to  $1 \times 10^{-5}$  M (5, 6), and even iodine is transportable by serum albumin (7). As confirmed recently (8, 9), cell-membrane TH transporters also carry TH precursors (MIT and/or DIT).

While iodinated Tyr are frequently part of the iodination consensus motifs (D/E, Y; S/T, Y, S; E, X, Y) (10), inconsistencies exist. For instance, the Asp-Tyr motif is associated with a complete iodination pattern (MIT, DIT, T3 and T4) in mY1310 but only T3 and T4 in the corresponding hY1309, which in turn is a pattern different from mY2586 (MIT and DIT) and from the corresponding hY2586 (MIT only). Even when any of the iodination motifs is present, one Tyr at the corresponding position in Tg from the two species may or may not be iodinated. This is, for instance, the case of mY235 (with this Tyr belonging to the stretch Ser-Gly-Tyr and being MIT-forming), while hTg235 also belongs to a Ser-Gly-Tyr stretch but it is non-iodinated.

In brief, we hypothesized that copies of the 5-residue core hydrophobic TH-binding motif are present in Tg, and that scrutiny of their sequence and position with respect to iodinated/non-iodinated Tyr residues might help explain why (i) only a limited number of Tyr are iodinated; (ii) of the iodinated Tyr, some form one or both iodotyrosines while others form one or both TH. Because the different number of iodinated Tyr and iodination patterns existing between Tg from two widely studied species (mice and humans) are known (10, 11), object of our investigation was murine Tg (mTg) and human Tg (hTg).

## 3. MATERIALS AND METHODS

We retrieved the amino acid sequences of murine and human Tg from the Entrez Protein database (<https://www.ncbi.nlm.nih.gov/protein>). In both cases, we selected the precursors (2768 amino acids each), identified in the database by the reference numbers AAB53204.1 (mTg) and NP\_003226.4 (hTg). Next, we used the software MotifFinder (12) to search for occurrences of the motif “F/Y/W, V/I/L/M, X, X, V/I/L/M” within the two sequences. In positions 1, 2 and 5 of the motif, we included all amino acids of the same groups of those of the original motif (3). As in our previous papers (1, 2, 3, 4, 13, 14), amino acid groups were grouped as follows: A/G (Ala/Gly), D/E/N/Q (Asp/Glu/Asn/Gln), F/Y/W (Phe/Tyr/Trp), H/K/R (His/Lys/Arg), I/L/M/V (Ile/Leu/Met/Val), S/T (Ser/Thr), C (Cys), P (Pro). We defined a full match with the above “F/Y/W, V/I/L/M, X, X, V/I/L/M” motif as “perfect”, while a match of only the first (F/Y/W) and second (V/I/L/M) position or a match of only the first (F/Y/W) and fifth position (V/I/L/M) was termed as “imperfect”.

## 4. RESULTS

Figure 1 and Figure 2 illustrate the positions of both perfect and imperfect TH-binding motifs in mTg and hTg, respectively. For hTg, this Figure also highlights such positions relative to the position of amino acids that have been reported to be mutated in patients with congenital hypothyroidism (15).

## Iodinated and non-iodinated tyrosines of thyroglobulin

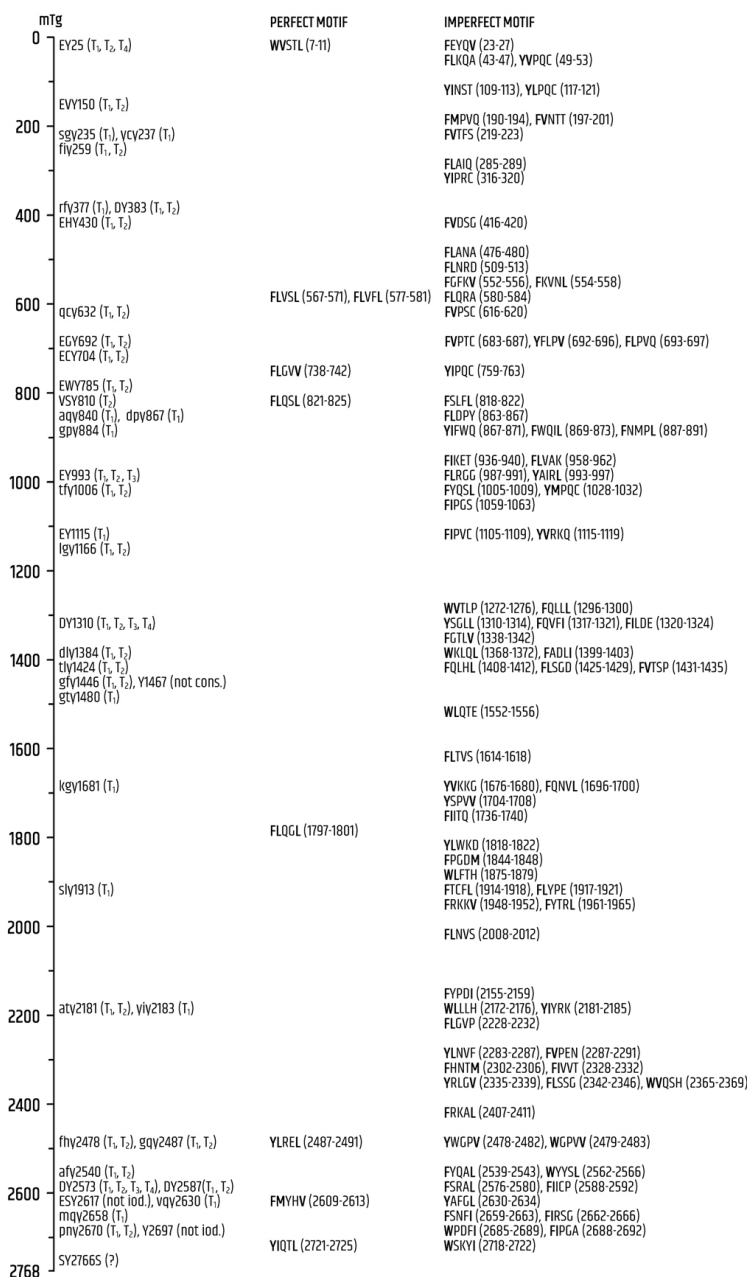


Figure 1. Positions of perfect and imperfect thyroid hormone-binding motifs in mouse Tg.

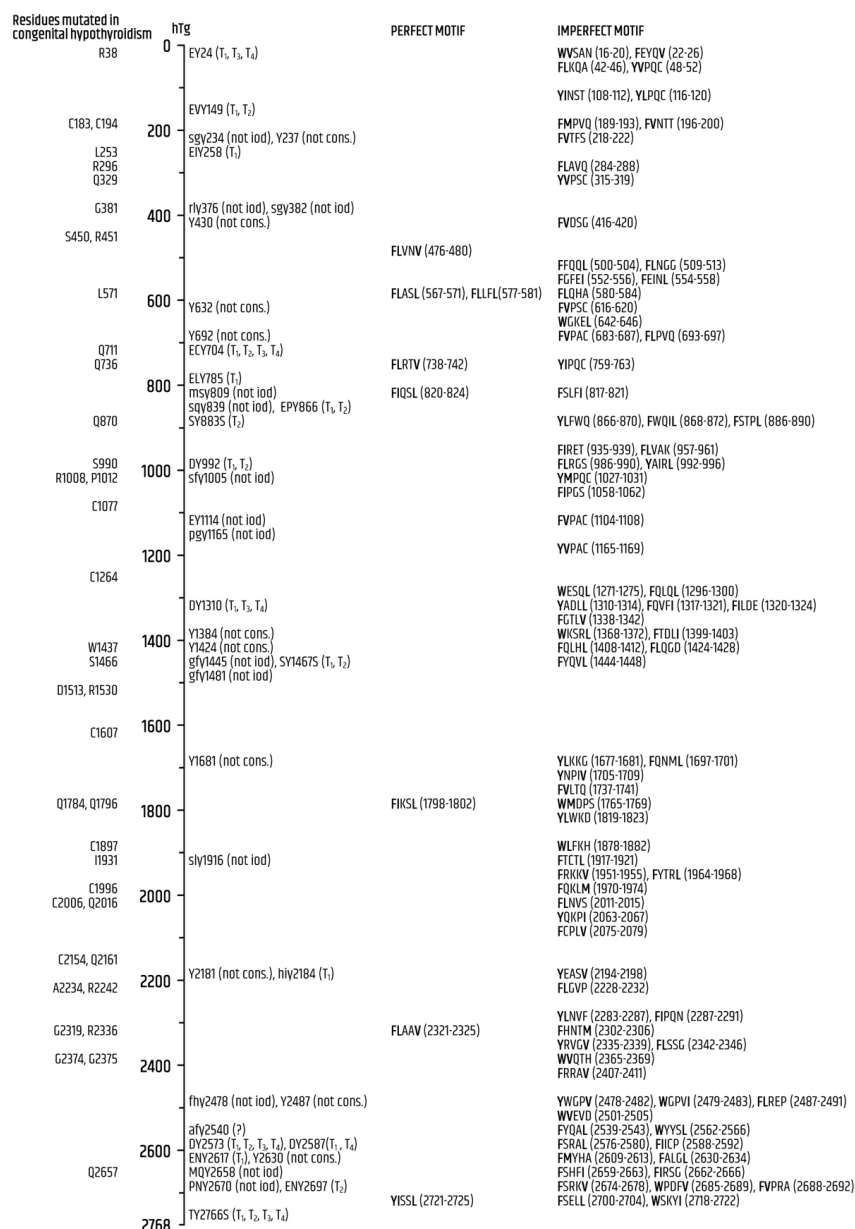
### 4.1. Perfect five-residue TH-binding motifs

There are nine copies of this motif in mTg, and eight in hTg, with six present in corresponding positions (Figure 3). In contrast, three perfect motifs of mTg (aa 7-11, 2487-2491 and 2609-2613) become imperfect at corresponding positions of hTg; the opposite occurs for two perfect motifs of hTg (aa 476-480 and 2321-2325) in corresponding positions of mTg (Figure 3).

Of the nine copies in mTg, five have Phe and Leu in the first and second position, and six

have Leu in the fifth (Figure 3). Of the eight copies in hTg, these patterns are observed in five and five copies, respectively. All 17 motifs are diverse, with not even two identical copies. The fifth position is at a very variable distance from the subsequent iodinated tyrosine, and similarly variable is the interval between the first position and the preceding iodinated tyrosine. For instance, in mTg there are 10 residues between Tyr810 and Phe821, 13 residues between Leu11 and Tyr25, whereas in hTg there are 217 residues between Tyr258 and Phe476 (Figure 3). Noteworthy, for Tyr forming T4 or both T4 and T3, the upstream or downstream perfect motif is relatively close.

## Iodinated and non-iodinated tyrosines of thyroglobulin



**Figure 2.** Positions of perfect and imperfect thyroid hormone-binding motifs in human Tg. Positions of amino acids that have been reported to be mutated in patients with congenital hypothyroidism (15) are shown on the left.

In either species Phe is consistently replaced by Trp in the most N-terminal motif (though in hTg the motif becomes imperfect) and by a noniodinated Tyr in the most C-terminal motif. Both changes are accompanied by the conservative replacement of Leu in the second position (Val N-terminally and Ile C-terminally). The Phe to Tyr replacement also occurs at position 2487 in mTg only. In addition to the motif at aa 2487-91, the motifs at aa 2609-2613 and 8-12 switch to imperfect in hTg. *Vice versa*, the motifs 476-480 and 2321-2325 in hTg switch to imperfect in mTg (Figure 3).

Of interest, four of the eight motifs in hTg are associated with hTg mutations that have been

reported in patients with congenital hypothyroidism (15). Precisely, Leu571 occupies the 5th position in the **FLASL** motif, while Gln736, Gln1796 and Gly2319 are always two positions ahead of the corresponding motifs **FLRTV**, **FIKSL** and **FLAAV** (Figure 3). Of these four motifs, three are associated with tyrosines that form MIT, DIT, T4 and T3 (Tyr704 and Tyr2573).

### 4.2. Imperfect five-residue TH-binding motifs

There are 84 copies of imperfect motifs in mTg (Figure 1) and 85 in hTg (Figure 2), with those nearest to iodinated tyrosines in mTg and their counterparts in hTg (n= 50 and 42) shown in Figure 4. Note that

Mouse Tg				Human Tg			
Position	Motif	Nearest iodinated tyrosines		Position	Motif	Nearest iodinated tyrosines	
		Upstream	Downstream			Upstream	Downstream
7-11	<b>WVSTL</b>	None	Y25 (M, D, T4)	16-20	<b>WVSAV</b>	None	Y24 (M, T4, T3)
476-480	<b>FLANA</b>	y430 (M, D)	y632 (M, D)	476-480	<b>FLVNV</b>	Y258 (M)	Y704 (M, D, T4, T3)
567-571	<b>FLVSL</b>	y430 (M, D)	y632 (M, D)	567-571	<b>FLASL</b> <sup>1</sup>	Y258 (M)	Y704 (M, D, T4, T3)
577-581	<b>FLVFL</b>	y430 (M, D)	y632 (M, D)	577-581	<b>FLFL</b>	Y258 (M)	Y704 (M, D, T4, T3)
738-742	<b>FLGVV</b>	y692 and Y704 (M, D)	Y785 (M, D)	738-742	<b>FLRTV</b> <sup>2</sup>	Y704 (M, D, T4, T3)	Y785 (M)
821-825	<b>FLQSL</b>	Y785 (M, D), <u>Y810</u> (D)	<u>Y840</u> , Y867, Y884 (M), Y993 (M, D, T3)	820-824	<b>FIQSL</b>	Y785 (M)	Y866 (M, D), Y883 (D), Y992 (M, D)
1797-1801	<b>FLQGL</b>	y1681 (M)	<u>Y1913</u> (M)	1798-1802	<b>FIKSL</b> <sup>2</sup>	Y1467 (M, D)	<b>Y2184</b> (M)
2321-2325	<b>ILAAV</b>	y2181 (M, D), <b>Y2183</b> (M)	<u>Y2478</u> (M, D)	2321-2325	<b>FLAAV</b> <sup>2</sup>	<b>Y2184</b> (M)	Y2540 (iodinated?), <b>Y2573</b> (M, D, T4, T3)
2487-2491	<b>YLREL</b> <sup>3</sup>	<u>Y2478</u> (M, D)	Y2540 (M, D)	2487-2491	<b>FLREP</b>	Y2184 (M)	Y2540 (iodinated?), <b>Y2573</b> (M, D, T4, T3)
2609-2613	<b>FMVHV</b>	<b>Y2573</b> (M, D, T4, T3), Y2587 (M, D)	y2630 and <u>Y2658</u> (M)	2609-2613	<b>FMVHA</b>	<b>Y2573</b> (M, D, T4, T3), Y2587 (M, T4)	<u>Y2617</u> (M)
2721-2725	<b>VIQTL</b>	<u>Y2670</u> (M, D)	Y2766 (uncertain <sup>4</sup> )	2721-2725	<b>VISSL</b>	Y2697 (D)	Y2766 (M, D, T4, T3)

**Figure 3.** Perfect 5-residue thyroid hormone-binding motifs in mouse Tg (mTg, accession number AAB53204.1) and human Tg (hTg, accession number NP\_003226.4), and their positions in relation with the nearest upstream and downstream iodinated tyrosines. Footnote: M=moniodotyrosine; D=diiodotyrosine. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Identical or conservatively replaced residues between the two species at any position are highlighted in **green** or **turquoise**, respectively. When in either species the motif is imperfect, it is typed *italicized*. Of the tyrosines of mTg appearing in this table, those not conserved in hTg are written in lower case (y). Underlined tyrosines in Tg of one species indicate that they become noniodinated in Tg of the other species. Corresponding tyrosines that form the same iodotyrosine(s) and/or iodothyronine(s) in the two species are typed **bold-faced** in columns 3, 4, 7 and 8 of this Table. <sup>1</sup>Residue L571 was found to be mutated in patients with congenital hypothyroidism due to Tg mutations. <sup>2</sup>Residues Q736, Q1796 and G2319, located very close to perfect 5-residue thyroid hormone binding motif, were found to be mutated in patients with congenital hypothyroidism due to Tg mutations. <sup>3</sup>The tyrosine in the motif **YLREL** is iodinated (M, D). <sup>4</sup>According to Dedieu *et al.* (11), "no definitive conclusion should be drawn" about the iodination status of Y2766 of mTg.

one motif can be placed simultaneously downstream with respect to one iodinated Tyr but upstream with respect to a subsequent iodinated Tyr. Similarly to the perfect motifs (see above), not even two imperfect motifs in either species are identical. However, a number of motifs in one species are maintained in the other species. For instance, in regard to mTyr150/hTyr149, in both species the motif is YLPQC upstream and FMPVQ downstream. Other examples are (i) mTyr704/hTyr704, with the upstream motif FLPVQ and the downstream motif YIPQC that are present in both species; (ii) mTyr2573/hTyr2573 with the upstream motif WYYSL and the downstream motif FSRAL; (iii) mTyr2587/hTyr2587 with the upstream motif FSRAL and the downstream motif FI/CP. mTyr150/hTyr149 form the same iodinated molecules (MIT and DIT), as do mTyr2573/hTyr2573 (MIT, DIT, T4 and T3). Instead, some difference exists for both Tyr704 (MIT and DIT in mTg, but MIT, DIT, T4 and T3 in hTg) and Tyr2587 (MIT and DIT in mTg, but MIT and T4 in hTg).

Phe is frequent in the first position of the motif, but starting from approximately the 1400th

residue, Phe is conservatively replaced by Trp. Similarly to the perfect motif, when the imperfect motif maintains the I/L/V/M residue in the fifth position, this residue is frequently Leu. When it does not and the motif falls in the cystein-rich domain of Tg, then the fifth position is frequently occupied by Cys (Figure 4). In mTg (and hTg), such motifs are **FVPSC**, **FVPTC**, **FIPVC** (**FVPAC**), **YIPQC** (**YIPQC**), **YLPQC** (**YLPQC**) or **YMPQC** (**YMPQC**). Interestingly, with no exceptions in mTg and only one exception in hTg (Tyr704), the Cys-ending imperfect motifs are never associated with a hormone-forming Tyr, but consistently with iodotyrosine(s)-forming Tyr (Figure 4).

Of the 14 noniodinated Tyr of hTg in Figure 4, six are associated with loss of the motif compared with presence of the motif in the corresponding position of mTg. *Vice versa*, of the two non-iodinated Tyr of mTg in Figure 4, one is associated with loss of the motif compared with presence of the motif in the corresponding position of hTg. This data, together with the aforementioned pattern of the Cys-ending imperfect motifs and the fact that some mutations of



# Iodinated and non-iodinated tyrosines of thyroglobulin

Mouse Tg	Nearest imperfect motif (position) <sup>1</sup>		Human Tg	Nearest imperfect motif (position) <sup>1</sup>	
Iodinated Tyr	Upstream	Downstream	Iodinated Tyr	Upstream	Downstream
Y25 (M, D, T4) <sup>2,3</sup>	<b>I</b> vaan (17-21)	<b>FLKQA</b> (43-47)	Y24 (M, T4, T3) <sup>2,3</sup>	<b>WV</b> SA <del>N</del> (16-20)	<b>FLKQA</b> (42-46)
Y150 (M, D) <sup>4</sup>	<b>YL</b> PQC (117-21)	<b>FMPVQ</b> (190-94)	Y149 (M, D) <sup>4</sup>	<b>YL</b> PQC (116-20)	<b>FMPVQ</b> (189-93)
Y235 (M)	<b>FVTFS</b> (219-23)	<b>FLAIQ</b> (285-9)	Y234 not iod.	<b>FVTFS</b> (218-22)	<b>FLAVQ</b> (284-88)
Y237 (M)	<b>FVTFS</b> (219-23)	<b>FLAIQ</b> (285-9)	Y237 not cons.		
Y259 (M, D) <sup>4</sup>	<b>FVTFS</b> (219-23)	<b>FLAIQ</b> (285-9)	Y258 (M) <sup>4</sup>	<b>FVTFS</b> (218-22)	<b>FLAVQ</b> (284-88)
Y377 (M)	<b>YIPRC</b> (316-20)	<b>FVDSG</b> (416-20)	Y376 not iod.	<b>YV</b> ASC (315-19)	<b>FVDSG</b> (416-20)
Y383 (M, D) <sup>5</sup>	<b>YIPRC</b> (316-20)	<b>FVDSG</b> (416-20)	Y382 not iod. <sup>8</sup>	<b>YV</b> ASC (315-19)	<b>FVDSG</b> (416-20)
Y430 (M, D) <sup>4</sup>	<b>FVDSG</b> (416-20)	<b>FLANA</b> (476-80)	Y430 not cons.		
Y632 (M, D)	<b>FVPSC</b> (616-20)	<b>FVPTC</b> (683-7)	Y632 not cons.		
Y692 (M, D) <sup>2,4</sup>	<b>FVPTC</b> (683-7)	<b>FLPVQ</b> (693-7)	Y692 not cons.		
Y704 (M, D) <sup>4</sup>	<b>FLPVQ</b> (693-7)	<b>YIPQC</b> (759-63)	Y704 (M, D, T4, T3) <sup>4</sup>	<b>FLPVQ</b> (693-97)	<b>YIPQC</b> (759-63)
Y785 (M, D) <sup>4</sup>	<b>YIPQC</b> (759-63)	<b>FSLFL</b> (818-22)	Y785 (M) <sup>4</sup>	<b>YIPQC</b> (759-63)	<b>FSLFI</b> (817-21)
Y810 (D)	<b>YIPQC</b> (759-63)	<b>FSLFL</b> (818-22)	Y809 not iod.	<b>YIPQC</b> (759-63)	<b>FSLFI</b> (817-21)
Y840 (M)	<b>FSLFL</b> (818-22)	<b>FLDPY</b> (863-7)	Y839 not iod.	<b>FSLFI</b> (817-21)	<b>Ilepy</b> (862-66)
Y867 (M) <sup>2</sup>	<b>FSLFL</b> (818-22)	<b>FWQIL</b> (869-73)	Y866 (M, D) <sup>2,4</sup>	<b>FSLFI</b> (817-21)	<b>FWQIL</b> (868-872)
Y884 (M)	<b>FWQIL</b> (869-873)	<b>FNMPLE</b> (887-91)	Y883 (D) <sup>6</sup>	<b>FWQIL</b> (868-72)	<b>FSTPL</b> (886-90)
Y993 (M, D, T3) <sup>2,3</sup>	<b>FLRGG</b> (987-991)	<b>FYQSL</b> (1005-9)	Y992 (M, D) <sup>2,5</sup>	<b>FLRGS</b> (986-90)	<b>fyqrr</b> (1004-8)
Y1006 (M, D) <sup>2</sup>	<b>YAIRL</b> (993-7)	<b>YMPQC</b> (1028-32)	Y1005 not iod.	<b>YAIRL</b> (992-96)	<b>YMPQC</b> (1027-31)
Y1115 (M) <sup>2,3</sup>	<b>FIPVC</b> (1105-9)	<b>WV</b> TLP (1272-6)	Y1114 not iod. <sup>3</sup>	<b>FVPAC</b> (1104-8)	<b>YVPAC</b> (1165-9)
Y1166 (M, D)	<b>YVRKQ</b> (1115-9)	<b>WV</b> TLP (1272-6)	Y1165 not iod. <sup>2</sup>	<b>yarlc</b> (1114-18)	<b>WESQL</b> (1271-5)
Y1310 (M, D, T4, T3) <sup>2,5</sup>	<b>FQLLL</b> (1296-300)	<b>FQVFI</b> (1317-21)	Y1310 (M, T4, T3) <sup>2,5</sup>	<b>FQLQL</b> (1296-300)	<b>FQVFI</b> (1317-21)
Y1384 (M, D)	<b>WKLQL</b> (1368-72)	<b>FADLI</b> (1399-403)	Y1384 not cons.		
Y1424 (M, D)	<b>FQLHL</b> (1408-12)	<b>FLSGD</b> (1425-9)	Y1424 not cons.		
Y1446 (M, D)	<b>FVTSP</b> (1431-5)	<b>WLQTE</b> (1552-6)	Y1445 not iod. <sup>2</sup>	<b>igtsp</b> (1430-4)	<b>wwetc</b> (1553-7)
Y1467 not cons.			Y1467 (M, D) <sup>6</sup>	<b>FYQVL</b> (1444-8)	<b>YLKKG</b> (1677-81)
Y1480 (M)	<b>FVTSP</b> (1431-5)	<b>WLQTE</b> (1552-6)	Y1481 not iod.	<b>igtsp</b> (1430-4)	<b>wwetc</b> (1553-7)
Y1681 (M)	<b>YVKKG</b> (1676-80)	<b>FQNVL</b> (1696-700)	Y1681 not cons.		
Y1913 (M)	<b>WLLTH</b> (1875-9)	<b>FTCFLE</b> (1914-8)	Y1916 not iod.	<b>WLFKH</b> (1878-82)	<b>FTCTL</b> (1917-21)
Y2181 (M, D) <sup>2</sup>	<b>WLLH</b> (2172-6)	<b>FLGVP</b> (2228-32)	Y2181 not cons.		
Y2183 (M) <sup>2</sup>	<b>icpsa</b> (2072-6)	<b>sdvts</b> (2193-7)	Y2184 (M)	<b>FCPLV</b> (2075-9)	<b>YEASV</b> (2194-8)
	<b>WLLH</b> (2172-6)	<b>FLGVP</b> (2228-32)		<b>rlllr</b> (2173-7)	<b>FLGVP</b> (2228-32)
Y2478 (M, D)	<b>FRKAL</b> (2407-11)	<b>WGPVV</b> (2479-83)	Y2478 not iod. <sup>2</sup>	<b>FRRAV</b> (2407-11)	<b>WGPVI</b> (2479-83)
Y2487 (M, D)	<b>WGPVV</b> (2479-83)	<b>FYQAL</b> (2539-43)	Y2487 not cons.		
Y2540 (M, D) <sup>2</sup>	<b>WGPVV</b> (2479-83)	<b>WYYSL</b> (2562-6)	Y2540 (iod.?) <sup>2</sup>	<b>WVEVD</b> (2501-5)	<b>WYYSL</b> (2562-6)
Y2573 (M, D, T4, T3) <sup>5</sup>	<b>WYYSL</b> (2562-6)	<b>FSRAL</b> (2576-80)	Y2573 (M, D, T4, T3) <sup>5</sup>	<b>WYYSL</b> (2562-6)	<b>FSRAL</b> (2576-80)
Y2587 (M, D) <sup>5</sup>	<b>FSRAL</b> (2576-80)	<b>FIICP</b> (2588-92)	Y2587 (M, T4) <sup>5</sup>	<b>FSRAL</b> (2576-80)	<b>FIICP</b> (2588-92)
Y2617 not iod. <sup>4</sup>	<b>FMYHV</b> (2609-13)	<b>YAFGL</b> (2630-4)	Y2617 (M) <sup>4</sup>	<b>FMYHA</b> (2609-13)	<b>FALGL</b> (2630-34)
Y2630 (M) <sup>2</sup>	<b>FIICP</b> (2588-92)	<b>FSNFI</b> (2659-63)	Y2630 not cons.		
Y2658 (M)	<b>YAFGL</b> (2630-4)	<b>FSNFI</b> (2659-63)	Y2658 not iod.	<b>FALGL</b> (2630-4)	<b>FSHFI</b> (2659-63)
Y2670 (M, D)	<b>FIRSG</b> (2662-6)	<b>WPDFI</b> (2685-9)	Y2670 not iod.	<b>FIRSG</b> (2662-6)	<b>FSRKV</b> (2674-8)
Y2697 not iod. <sup>4</sup>	<b>FIPGA</b> (2688-92)	<b>lsaq</b> (2700-4)	Y2697 (D) <sup>4</sup>	<b>FVPR</b> (2688-92)	<b>FSELL</b> (2700-4)
Y2766 (uncertain) <sup>6</sup>	<b>WSKYI</b> (2718-22)	None (Tg ends at 2768)	Y2766 (M, D, T4, T3) <sup>7</sup>	<b>WSKYI</b> (2718-22)	None (Tg ends at 2768)

**Figure 4.** Imperfect 5-residue thyroid hormone-binding motifs in mouse Tg (mTg) and human Tg (hTg) that are located closest to iodinated tyrosines. Footnote: cons.=conserved; iod.=iodinated; M=moniodotyrosine; D=diiodotyrosine. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Identical or conservatively replaced residues between the two species at any position are highlighted in **green** or **turquoise**, respectively. Imperfect motifs are typed *italicized*. In hTg, a position is **underlined** and **boldfaced** to indicate that one of the five residues in the motif was reported to be mutated in cases of congenital hypothyroidism associated with Tg mutations (Q870, S990). The position is **underlined** only, when such mutation occurs very close to the motif (C194, R296, A2234, Q2657). Other mutations (C1264, W1437, C2006, Q2016, R2336, G2374, G2375) occurred very close to imperfect motifs not shown in this table. <sup>1</sup>When a 5-residue motif in one species loses the sequence to qualify as a motif in the corresponding position of the other species, the said sequence is reported in the table, but printed lower case on gray background. When the imperfect motif is conserved, but the nearest motif is different between mTg and hTg, position numbers in hTg are written in **red**. <sup>2</sup>The tyrosine is part of a motif. Consensus motifs for iodinated tyrosines: <sup>3</sup>EY, <sup>4</sup>EXY, <sup>5</sup>DY, <sup>6</sup>SYS, <sup>7</sup>TYS. <sup>8</sup>No consensus motif for iodination.

hTg causing congenital hypothyroidism occur within or very close to an imperfect motif (Figure 4), stimulated us to analyze in greater detail the characteristics of the motifs in relation to the iodination properties of tyrosines, including the type of iodotyrosine(s)/iodothyronine(s) formed (see below).

#### 4.3. Perfect/imperfect motifs in relation to tyrosines that, in both mTg and hTg, form solely MIT

We started by searching for analogies between tyrosines of mTg and hTg that form only MIT, taking note not only of the closest upstream and downstream TH-binding motifs but also of the iodination consensus motifs (Figure 5). There are 13 such Tyr in mTg, but only four in hTg, with only one being conserved in the two species (mY2183/hY2184). A remarkable difference is that only one mTyr (Y1115) has a iodination consensus motif (Asp-Tyr). In contrast, 3/4 hTyr (Y258, Y785 and Y2617) have another iodination consensus motif (Asp-X-Tyr).

Starting inspection of Figure 5 from the Tyr that is conserved in the two species (mTyr2183/hTyr2184), neither has any iodination consensus motif. We also notice that, except for the Ile preceding the Arg-Lys following this Tyr, there is no similarity either upstream or downstream of mTyr 2183/hTyr2184. Marked differences also include the upstream and downstream 5-residue imperfect motif and distance from Tyr 2183/2184. When analysis is performed within species, no pattern emerges. In mTg, the upstream 5-residue motif (which is of the perfect type for three tyrosines) is variably positioned (from zero to almost 60 residues ahead of Tyr2183), and so is the downstream motif occupying columns 22-26 in Figure 5 (from approximately 15 to over 150 residues away), with only the first position (Phe) being relatively common. A similar pattern applies to hTg, though the downstream motif at columns 22-26 is less variably positioned from the selectively MIT-forming Tyr (9 to 31 residues away). In six instances for mTg and none for hTg, the selectively MIT-forming Tyr is part of (Y867, Y1115 and Y2630) or immediately followed by an imperfect motif (Y884, Y1913 and Y2658).

#### 4.4. Perfect/imperfect motifs in relation to tyrosines that, in both mTg and hTg, form solely DIT

There are only one such Tyr in mTg, and two in hTg (Figure 6). mTyr810 had no iodination consensus motif, while hTyr883 and hTyr2697 had two different motifs (Ser-Tyr-Ser and Glu-X-Tyr). Interestingly, for Y2697, the X residue is Asn, exactly as for the selectively MIT-forming Y2617 (Figure 5). To add to the parallelism between Y2697 and Y2617 of hTg, similar is the number of residues between the fifth position of the upstream TH-binding motif (four and three), the sequence of such motif (EVPRA and

EMYHA, with underlined residues indicating identity or conservative replacements). However, the number of residues between the DIT-forming Tyr at the first position of the TH-binding motif at columns 22-26 (20 and 12), and the sequence of such motif differ substantially. Furthermore, the fourth and fifth position of the imperfect motif WSKYI coincide to be the first and the second position of the perfect motif YISSL (Figure 6). This mixture of imperfect and perfect motifs at columns 22-29, with Ser occupying column 23 and Ser-Leu occupying columns 28-29, plus Pro occupying the third position in the imperfect upstream motif (column 3) and Arg/Lys-Glu immediately following the DIT-forming tyrosines are the analogies between Y810 of mTg and Y2697 of hTg (Figure 6).

Finally, only Y2697 (as well as Y883) is immediately followed by another imperfect motif (FSELL), a fact that does not occur for any of the four MIT-forming tyrosines of hTg (Figure 5).

#### 4.5. Perfect/imperfect motifs in relation to tyrosines that, in both mTg and hTg, form only MIT and DIT

There are 19 such tyrosines in mTg and four in hTg, of which only the most N-terminal one (Y150) at a position matching mTg (Figure 7). Two mTyr (Y383 and Y2587) and one hTyr (Y992) have the Asp-Tyr consensus iodination motif. Another six mTyr (Y150, Y259, Y430, Y692, Y704 and Y785) and another two hTyr (Y149 and Y866) have eight different copies of the Glu-X-Tyr iodination motif, in that the X residue differs in each of the eight sequences. The remaining 11 mTyr have no iodination consensus motif, while the remaining hTyr1467 have the Ser-Tyr-Ser iodination motif.

In hTg, the iodinated Tyr is preceded, with an interval of one to 41 residues, by the fifth position of the upstream thyroid hormone binding motif, which is imperfect except for Tyr866. Tyr866 is part of a composite imperfect/perfect motif, while Tyr992 is part of an imperfect motif. The fifth position (Q870) in the downstream imperfect motif that includes Y866, the fifth position (Ser990) in the imperfect motif upstream of Y992, and the first Ser (S1466) of the Ser-Tyr-Ser that includes Y1467 are all positions where mutations associated with congenital hypothyroidism have been reported. Other residues mutated in congenital hypothyroidism (C183 and C194) flank the 5-residue TH-binding motif downstream of Y149. The motifs at columns 22-26 downstream of the four selectively DIT-forming Tyr of hTg have a relative abundance of Tyr in the first position, Met in the second position, Pro in the third or fourth position, these being characteristics that are not observed for the selectively MIT-forming hTyr (Figure 5) or selectively DIT-forming hTyr (Figure 6).

In mTg, these characteristics at columns 22-26 are more tenuous, with Tyr in the first position,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 219-298	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	9	S	G	Y235	<b>C</b>	Y	<b>C</b>	A	D	S	Q	G
mTg, 219-298	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	11	Y <sup>1</sup>	<b>C</b>	Y237	<b>C</b>	A	D	S	Q	G	R	E
mTg, 316-428	<b>Y</b>	<b>I</b>	<b>P</b>	<b>R</b>	<b>C</b>	54	R	F	Y377	F	E	T	P	D	Y <sup>2</sup>	F	S
mTg, 821-875	<b>F</b>	<b>L</b>	<b>Q</b>	<b>S</b>	<b>L</b>	12	A	Q	Y840	P	S	L	Q	D	V	P	Q
mTg, 821-899	<b>F</b>	<b>L</b>	<b>Q</b>	<b>S</b>	<b>L</b>	39	D <sup>3</sup>	P <sup>3</sup>	<b>Y867<sup>3</sup></b>	<b>I</b>	<b>F</b>	<b>W</b>	<b>Q</b>	<b>I</b>	<b>L</b>	<b>N</b>	<b>G</b>
mTg, 869-948	<b>F</b>	<b>W</b>	<b>Q</b>	<b>I</b>	<b>L</b>	8	G	P	Y884	S	D	<b>F</b>	<b>N</b>	<b>M</b>	<b>P</b>	<b>L</b>	<b>E</b>
mTg, 1105-1284	<b>F</b>	<b>I</b>	<b>P</b>	<b>V</b>	<b>C</b>	3	G	E	<b>Y1115</b>	<b>V</b>	<b>R</b>	<b>K</b>	<b>Q</b>	<b>T</b>	<b>S</b>	<b>G</b>	<b>T</b>
mTg, 1431-1564	<b>F</b>	<b>V</b>	<b>T</b>	<b>S</b>	<b>P</b>	42	G	T	Y1480	Q	E	Q	A	G	S	S	A
mTg, 1676-1708	<b>Y</b>	<b>V</b>	<b>K</b>	<b>K</b>	<b>G</b>				Y1681	E	S	T	A	A	G	Q	K
mTg, 1875-1960	<b>W</b>	<b>L</b>	<b>F</b>	<b>T</b>	<b>H</b>	31	S	L	Y1913	<b>F</b>	<b>T</b>	<b>C</b>	<b>F</b>	<b>L</b>	<b>Y</b>	<b>P</b>	<b>E</b>
mTg, 2172-2240	<b>W</b>	<b>L</b>	<b>L</b>	<b>L</b>	<b>H</b>	4	Y <sup>2</sup>	I	Y2183	R	K	S	G	I	P	L	V
mTg, 2609-2671	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>V</b>	14	V	Q	<b>Y2630</b>	A	F	G	<b>L</b>	P	F	Y	S
mTg, 2630-2697	<b>Y<sup>1</sup></b>	A	F	G	<b>L</b>	21	M	Q	Y2658	<b>F</b>	<b>S</b>	<b>N</b>	<b>F</b>	<b>I</b>	<b>R</b>	<b>S</b>	<b>G</b>
hTg, 218-296	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	33	E	I	Y258	D	T	I	F	A	G	L	D
hTg, 759-829	<b>Y</b>	<b>I</b>	<b>P</b>	<b>Q</b>	<b>C</b>	19	E	L	Y785	Q	R	W	E	A	Q	N	K
hTg, 2075-2206	<b>F</b>	<b>C</b>	<b>P</b>	<b>L</b>	<b>V</b>	102 <sup>4</sup>	H	I	Y2184	R	K	P	G	I	S	L	L
hTg, 2608-2642	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>A</b>	P	E	N	Y2617	G	H	G	S	L	E	L	L

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 219-298	R	E	L	38	<b>F</b>	<b>L</b>	A	I	Q	L	V	I	S	G	R	F	R
mTg, 219-298	L	A	E	36	<b>F</b>	<b>L</b>	A	I	Q	L	V	I	S	G	R	F	R
mTg, 316-428	P	Q	D	27	<b>F</b>	<b>V</b>	D	S	G	L	L	R	S	I	A	V	E
mTg, 821-875	V	V	L	11	<b>F</b>	<b>L</b>	D	P	Y <sup>1</sup>	<b>I</b>	<b>F</b>	<b>W</b>	<b>Q</b>	<b>I</b>	<b>L</b>	<b>N</b>	<b>G</b>
mTg, 821-899	Q	L	S	8	<b>F</b>	<b>N</b>	<b>M</b>	<b>P</b>	<b>L</b>	E	H	F	N	L	R	S	<b>C</b>
mTg, 869-948	H	F	N	40	<b>F</b>	<b>I</b>	K	E	T	E	E	I	V	S	A	S	N
mTg, 1105-1284	G	T	W	145	<b>W</b>	<b>V</b>	T	L	P	P	P	R	A	<b>C</b>	Q	R	P
mTg, 1431-1564	<b>C</b>	I	P	60	<b>W</b>	<b>L</b>	Q	T	E	A	G	L	S	E	S	Q	<b>C</b>
mTg, 1676-1708	S	F	E	3	<b>F</b>	<b>Q</b>	N	V	<b>L</b>	S	G	L	<b>Y</b>	<b>S</b>	<b>P</b>	<b>V</b>	<b>V</b>
mTg, 1875-1960	A	Q	V	23	<b>F</b>	<b>R</b>	R	K	<b>V</b>	V	L	N	D	R	V	K	N
mTg, 2172-2240	Q	S	D	33	<b>F</b>	<b>L</b>	G	V	P	Y	A	A	P	P	L	A	D
mTg, 2609-2671	A	Y	Q	17	<b>F</b>	<b>S</b>	N	F	<b>I</b>	R	S	G	N	P	N	Y <sup>2</sup>	P
mTg, 2630-2697	N	P	N	15	<b>W</b>	<b>P</b>	D	<b>F</b>	<b>I</b>	P	G	A	G	G	E	S	Y
hTg, 218-296	L	P	S	14	<b>F</b>	<b>L</b>	A	V	Q	S	V	I	S	G	R	F	R <sup>3</sup>
hTg, 759-829	G	Q	D	20	<b>F</b>	<b>S</b>	L	<b>F</b>	<b>I</b>	Q	<b>S</b>	<b>L</b>	Y	E	A	G	Q
hTg, 2075-2206	S				<b>Y</b>	<b>E</b>	A	S	<b>V</b>	P	S	V	P	I	S	T	H
hTg, 2608-2642	A	D	V	Q	<b>F</b>	<b>A</b>	L	G	<b>L</b>	P	F	Y	P	A	Y	E	G

**Figure 5.** Amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines that form MIT only (column 9) and their closest upstream and downstream TH-binding motifs. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>In position other than column 9, also this tyrosine forms MIT. <sup>2</sup>These tyrosines of mTg form both MIT and DIT: Y383, Y2170, Y2181.

<sup>3</sup>These amino acids are the terminal part of the imperfect motif FDLPY (863-867).

<sup>4</sup>Amino acids R296, C2154 and Q2161 were reported to be mutated in cases of congenital hypothyroidism associated with Tg mutations.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 759-830	<b>Y</b>	<b>I</b>	<b>P</b>	<b>Q</b>	<b>C</b>	44	V	S	Y810	R	E	V	A	S	R	N	
hTg, 868-947	<b>F</b>	<b>W</b>	<b>Q</b> <sup>1</sup>	<b>I</b>	<b>L</b>	8	G	S	Y883	S	D	<b>F</b>	<b>S</b>	<b>T</b>	<b>P</b>	<b>L</b>	A
hTg, 2688-2730	<b>F</b>	<b>V</b>	<b>P</b>	<b>R</b>	<b>A</b>	2	E	N	Y2697	K	E	<b>F</b>	<b>S</b>	<b>E</b>	<b>L</b>	<b>L</b>	P
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 759-830					<b>F</b>	<b>S</b>	<b>L</b>	<b>F</b>	<b>L</b>	<b>Q</b>	<b>S</b>	<b>L</b>	<b>Y</b>	D	A	G	Q
hTg, 868-947	H	F	D	40	<b>F</b>	<b>I</b>	R	E	T	E	E	I	V	S	A	S	N
hTg, 2688-2730	N	R	Q	9	<b>W</b>	<b>S</b>	K	<b>Y</b>	<b>I</b>	<b>S</b>	<b>S</b>	<b>L</b>	K	T	S	A	D

**Figure 6.** Amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines that form DIT only (column 9) and their closest upstream and downstream TH-binding motifs. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>Amino acid Q870 was reported to be mutated in cases of congenital hypothyroidism associated with Tg mutations.

Met in the second and Pro in the third or fourth position occurring with corresponding frequencies of 2/19, 3/19 and 4/19; the fifth position is Cys in 6/19 cases. Columns 22-26 downstream of Y708 and Y785 are occupied by the same perfect motif. Six Tyr are part of a perfect (Y2487) or imperfect motif (Y692, Y1006, Y2181, Y2478 and 2540), and another Tyr (Y2587) precedes an imperfect motif just immediately. Phe, Tyr or Trp at column 22 are located six to 105 residues away from Tyr at column 9. Interestingly, this distance is of the same magnitude for pairs of Tyr, namely 14 residues for Y1384 and Y2670, 21 residues for Y2540 and Y2487, 45 residues for Y430 and Y704, and 105 residues for Y1166 and Y1446.

Starting from Y1006, whenever the first position of the upstream motif is Tyr, this Tyr is either MIT- and DIT-forming (Y2478 and Y2487) or MIT-, DIT- and T3-forming (Y993) (Figure 7). With one exception (Y2540), the upstream motif is always imperfect, and with Pro occupying the third or the fourth position in 8/19 cases. In 12/19 cases, there is an interval of two to 11 residues between the fifth position of the upstream motif and the selectively DIT-forming mTyr.

#### 4.6. Perfect/imperfect motifs in relation to tyrosines that, in both mTg and hTg, form MIT, DIT, T4 and T3

There are two such Tyr in mTg, and three in hTg, of which only one (Tyr2573) is shared by mTg and hTg (Figure 8). In addition, it is uncertain whether the mTg homologue of hTyr2766 is iodinated. Thus, mTg sequence 2761-2768 is included in Figure 8 (and compared with other MIT-, DIT-, T4- and T3-forming tyrosines) to ascertain whether data are in favor or against iodination of mTyr2766.

While mTyr1310 and mTyr2573 have the Asp-Tyr iodination motif, only hTyr2573 has; hTyr704

has the Glu-X-Tyr iodination motif, but hTyr2766 has the Thr-Tyr-Ser iodination motif, which is homologous to the Ser-Tyr-Ser motif of mTyr2766. The homology between the two species around Tyr2766 is reinforced by the last residue (Lys), by the same number of residues (n= 40) between Tyr2766 and the fifth position of the upstream motif, and by the perfect nature of the motif. Forty is a number greater than the corresponding number of 6 or 9 residues for the other tyrosines in Figure 8. Furthermore, the first, second, and fifth position of this motif are occupied by identical residues, and the fourth by conservatively similar residues. The same residues in this fourth and fifth position (Ser-Leu) and a conservatively similar residue (Trp) are present in the motif upstream of both mTyr2573 and hTyr2573. All these observations enhance the chances that mTyr2566 be indeed iodinated and even sharing MIT-, DIT-, T4- and T3-formation with hTyr2766. No comment on the downstream motif is possible because both mTg and hTg end with Lys2768.

In addition to the downstream motif at columns 22-26, mTyr1310 is part of an imperfect motif, which is followed by another imperfect motif at columns 16-20. mTyr2573 and hTyr2573 are followed by an imperfect motif at aa 12-73. The first position of all motifs downstream of all tyrosines in Figure 8 is consistently occupied by Phe.

Upon comparison of mTg 2562-2600 with hTg 2562-2600, the four residues in column 6 (EHST) and the three in column 21 (RDY) are identical in the two species. Strikingly enough, differences start immediately before the upstream motif (columns 1-5) and immediately after the downstream motif (columns 22-26). Indeed, the residue preceding W in column 1 is V in mTg and T in hTg; as shown in Figure 8, the residue following the fifth position of the downstream motif is M in mTg and I in hTg.

# Iodinated and non-iodinated tyrosines of thyroglobulin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 117-202	<b>Y</b>	<b>L</b>	P	Q	<b>C</b>	26	E	V	Y150	G	T	R	Q	Q	G	R	P
mTg, 219-297	<b>F</b>	<b>V</b>	T	F	S	33	E	I	Y259	D	T	I	F	A	G	L	D
mTg, 316-428	<b>Y</b>	<b>I</b>	P	R	<b>C</b>	60	P	D	Y383	F	S	P	Q	D	L	L	S
mTg, 416-488	<b>F</b>	<b>V</b>	D	S	G	7	E	H	Y430	Q	R	L	S	E	S	R	S
mTg, 616-695	<b>F</b>	<b>V</b>	P	S	<b>C</b>	9	Q	<b>C</b>	Y632	A	G	E	<b>C</b>	W	<b>C</b>	V	D
mTg, 683-750	<b>F</b>	<b>V</b>	P	T	<b>C</b>	2	E	G	<b>Y692</b>	<b>F</b>	<b>L</b>	P	<b>V</b>	Q	<b>C</b>	F	N
mTg, 693-750	<b>F</b>	<b>L</b>	P	V	Q	4	E	<b>C</b>	Y704	<b>C</b>	V	D	T	E	G	Q	V
mTg, 759-830	<b>Y</b>	<b>I</b>	P	Q	<b>C</b>	19	E	W	Y785	E	R	W	K	T	Q	N	G
mTg, 993-1040	<b>Y</b> <sup>1</sup>	A	I	R	<b>L</b>	6	T	<b>F</b>	Y1006	Q	S	<b>L</b>	R	A	S	L	G
mTg, 1105-1284	<b>Y</b>	<b>V</b>	R	K	Q	44	L	G	Y1166	S	P	V	C	E	A	L	D
mTg, 1368-1411	<b>W</b>	K	L	Q	<b>L</b>	9	D	L	Y1384	S	I	E	R	A	V	T	G
mTg, 1408-1443	<b>F</b>	Q	L	H	<b>L</b>	9	T	L	Y1424	<b>F</b>	<b>L</b>	S	G	D	S		
mTg, 1431-1564	<b>F</b>	<b>V</b>	T	S	P	8	G	F	Y1446	R	V	P	T	T	R	Q	D
mTg, 2172-2240	<b>W</b>	<b>L</b>	L	L	H	2	A	T	<b>Y2181</b>	<b>I</b>	Y <sup>2</sup>	R	K	S	G	I	P
mTg, 2407-2499	<b>F</b>	R	K	A	<b>L</b>	64	F	H	<b>Y2478</b>	<b>W</b>	G	P	<b>V</b>	<b>V</b>	D	G	Q
mTg, 2478-2551	<b>Y</b> <sup>3</sup>	W	G	P	<b>V</b>	2	G	Q	<b>Y2487</b>	<b>L</b>	R	E	<b>L</b>	P	S	R	R
mTg, 2487-2574	<b>Y</b> <sup>3</sup>	<b>L</b>	R	E	<b>L</b>	46	A	<b>F</b>	Y2540	Q	A	<b>L</b>	Q	N	S	L	G
mTg, 2576-2621	<b>F</b>	S	R	A	<b>L</b>	4	R	D	Y2587	<b>F</b>	<b>I</b>	<b>I</b>	<b>C</b>	P	M	V	N
mTg, 2662-2697	<b>F</b>	<b>I</b>	R	S	G	N	P	N	Y2670	P	H	E	F	S	R	K	A
hTg, 116-201	<b>Y</b>	<b>L</b>	P	Q	<b>C</b>	26	E	V	Y149	G	T	R	Q	L	G	R	P
hTg, 820-898	<b>F</b>	<b>I</b>	Q	S	<b>L</b>	39	E	P	<b>Y866</b>	<b>L</b>	<b>F</b>	W	Q <sup>5</sup>	I	<b>L</b>	N	G
hTg, 986-1039	<b>F</b>	<b>L</b>	R	G	S <sup>5</sup>			D	<b>Y992</b>	A	I	R	<b>L</b>	A	A	Q	S
hTg, 1444-1689	<b>F</b>	<b>Y</b>	Q	V	<b>L</b>	16	G	S <sup>5</sup>	Y1467	S	Q	D	E	E	<b>C</b>	I	P

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 117-202	T	R	<b>C</b>	28	<b>F</b>	<b>M</b>	P	V	Q	<b>C</b>	K	<b>F</b>	<b>V</b>	N	T	T	D
mTg, 219-297	Q	A	S	14	<b>F</b>	<b>L</b>	A	I	Q	L	V	I	S	G	R	F	R
mTg, 316-428	S	E	D	21	<b>F</b>	<b>V</b>	D	S	G	L	L	R	S	I	A	V	E
mTg, 416-488	L	L	R	34	<b>F</b>	<b>L</b>	A	N	A	A	Q	F	N	L	S	G	A
mTg, 616-695	S	R	G	39	<b>F</b>	<b>V</b>	P	T	<b>C</b>	T	R	E	G	Y <sup>3,6</sup>	F <sup>6</sup>	L <sup>6</sup>	P <sup>6</sup>
mTg, 683-750	S	E	<b>C</b>	34	<b>F</b>	<b>L</b>	G	V	<b>V</b>	G	V	L	L	S	N	S	S
mTg, 693-750	I	P	G	22	<b>F</b>	<b>L</b>	G	V	<b>V</b>	G	V	L	L	S	N	S	S
mTg, 759-830	D	G	Q	21	<b>F</b>	S	L	<b>F</b>	<b>L</b>	Q	S	<b>L</b>	Y	A	D	G	Q
mTg, 993-1040	K	S	D	10	<b>Y</b>	<b>M</b>	P	Q	<b>C</b>	N	M	I	G	G	W	E	P
mTg, 1105-1284	G	A	F	94	<b>W</b>	<b>V</b>	T	L	P	P	P	R	A	<b>C</b>	Q	R	P
mTg, 1368-1411	Q	D	L	3	<b>F</b>	A	D	L	<b>I</b>	Q	S	G	R	F <sup>7</sup>	Q <sup>7</sup>	L <sup>7</sup>	H <sup>7</sup>
mTg, 1408-1443					<b>F</b>	<b>V</b>	T	S	P	G	L	S	L	D	V	L	E
mTg, 1431-1564	A	L	G	94	<b>W</b>	<b>L</b>	Q	T	E	A	G	L	S	E	S	Q	<b>C</b>
mTg, 2172-2240	L	V	Q	35	<b>F</b>	<b>L</b>	G	V	P	Y	A	A	P	P	L	A	D
mTg, 2407-2499					<b>Y</b> <sup>3</sup>	<b>L</b>	R	E	<b>L</b>	P	S	R	R	L	K	R	P
mTg, 2478-2551	L	K	R	40	<b>F</b>	Y	Q	A	<b>L</b>	Q	N	S	L	G	G	E	D
mTg, 2487-2574	G	E	D	10	<b>W</b>	Y	Y	S	<b>L</b>	E	H	S	T	D	D	Y <sup>4</sup>	A
mTg, 2576-2621	M	A	S	10	<b>F</b>	<b>M</b>	Y	H	<b>V</b>	P	E	S	Y	G	H	G	S
mTg, 2662-2697	A	E	F	3	<b>W</b>	P	D	<b>F</b>	<b>I</b>	P	G	A	G	G	E	S	Y
hTg, 116-201	K	R	<b>C</b>	28 <sup>5</sup>	<b>F</b>	<b>M</b>	P	V	Q	<b>C</b> <sup>5</sup>	K	<b>F</b>	<b>V</b>	N	T	T	D
hTg, 820-898	Q	L	S	8	<b>F</b>	S	T	P	<b>L</b>	A	H	F	D	L	R	N	<b>C</b>
hTg, 986-1039	T	L	S	23	<b>Y</b>	<b>M</b>	P	Q	<b>C</b>	D	A	F	G	S	W	E	P
hTg, 1444-1689	<b>C</b>	P	V	198 <sup>5</sup>	<b>Y</b>	<b>L</b>	K	K	G	Q	G	S	T	T	T	L	Q

**Figure 7.** Amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines that form both MIT and DIT only (column 9) and their closest upstream and downstream TH-binding motifs. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>mTg Y993 forms MIT, DIT and T3. <sup>2</sup>These tyrosines form MIT only. <sup>3</sup>These tyrosines of mTg form both MIT and DIT. <sup>4</sup>These tyrosines form MIT, DIT, T4 and T3. <sup>5</sup>Amino acids C183, C194, Q870, S990, S1466, R1530, D1513, C1607 were reported to be mutated in cases of congenital hypothyroidism associated with Tg mutations. <sup>6</sup>These amino acids are the initial part of the imperfect motif YFLPV (692-696). <sup>7</sup>These amino acids are the initial part of the imperfect motif FQLHL (1408-1412).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 1296-1350	<b>F</b>	Q	L	L	<b>L</b>	7	V	D	<b>Y1310</b>	S	G	L	<b>L</b>	Q	A	<b>F</b>	Q
mTg, 2562-2600	<b>W</b>	Y	Y	S	<b>L</b>	4	D	D	Y2573	A	S	<b>F</b>	S	R	A	<b>L</b>	E
mTg, 2721-2768	<b>Y</b>	<b>I</b>	Q	<b>T</b>	<b>L</b>	38	K	S	Y2766 (?) <sup>1</sup>	S	K						
hTg, 693-750	<b>F</b>	<b>L</b>	P	V	Q	4	E	<b>C</b>	Y704	<b>C</b>	V	D	A	E	G	Q <sup>2</sup>	A
hTg, 2562-2600	<b>W</b>	Y	Y	S	<b>L</b>	4	D	D	Y2573	A	S	<b>F</b>	S	R	A	<b>L</b>	E
hTg, 2721-2768	<b>Y</b>	<b>I</b>	S	S	<b>L</b>	38	K	T	Y2766	S	K						
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 1296-1350	V	F	<b>I</b>	16	<b>F</b>	G	T	<b>L</b>	<b>V</b>	S	S	T	V	<b>C</b>	D	N	S
mTg, 2562-2600	N	A	T	3	<b>F</b>	<b>I</b>	<b>I</b>	<b>C</b>	P	M	V	N	M	A	S	L	W
mTg, 2721-2768																	
hTg, 693-750	I	P	G	22 <sup>1</sup>	<b>F</b>	<b>L</b>	R	T	<b>V</b>	Q	A	L	L	S	N	S	S
hTg, 2562-2600	N	A	T	3	<b>F</b>	<b>I</b>	<b>I</b>	<b>C</b>	P	I	I	D	M	A	S	A	W
hTg, 2721-2768																	

**Figure 8.** Amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines that form MIT, DIT, T4 and T3 (column 9) and their closest upstream and downstream TH-binding motifs. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match with those at first, second or fifth position of the motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>It is uncertain whether the mTg homologue of hTyr2766 is iodinated. <sup>2</sup>Q711X (Q692X) and Q736X (Q717X) mutations of hTg.

#### 4.7. Perfect/imperfect motifs in relation to tyrosines that are iodinated in Tg from one species but not iodinated in Tg from the other species

##### 4.7.1. Forming solely MIT, and only in one species

Tyrosines that are MIT-forming only in mTg, but non-iodinated in hTg, are the first six in Figure 9. In contrast, the last Tyr (Tyr2617) in Figure 9 is MIT-forming in hTg but non-iodinated in mTg.

Iodination consensus motifs cannot account for the said difference in iodination between corresponding tyrosine residues in the two Tg species. Indeed, only one of the top six mouse Tyr in column 9 of Figure 9 (Y1115) has one of the known iodination motifs (E-Y). However, the non-iodinated Tyr1114 also has the E-Y iodination motif.

In contrast, the TH-binding motif could help explain why a given Tyr is iodinated and its counterpart in the other species is not. mTyr1115 has Tyr in the first position of an imperfect TH-binding motif, with Val in the corresponding second position (column 10 in Figure 9). Remarkably, the second position is occupied by a different amino acid (Ala) in the corresponding position of hTg. A change in the second position also occurs in the motif (columns 1-5) upstream not only of hTyr1114 but also of hTyr376 and hTyr1481. In the motif upstream of hTyr2658 and hTyr1916 the change concerns the third and the fourth position, with the fourth position also being affected concerning hTyr376. The loss of iodination of mTyr2617, compared to iodinated hTyr2617, is associated with a change in

the fifth position of the upstream motif. Concerning the downstream motif at columns 22-26, a change in at least one of the five positions occurs for four hTyr (hTyr234, hTyr1114, hTyr1481, hTyr1916) as well as for mTyr2617; the third and the second position are those most frequently affected. Downstream of hTg2658, the change in the motif is quite drastic, in that differences in primary structure between mTg and hTg create an imperfect TH-binding motif (FSRKV) that is absent in mTg. Furthermore, this new motif is the closest to hTyr2658, with an interval of 15 residues, compared to the interval of 26 residues of mTyr2658.

Peculiar changes at positions intermediated between Tyr at position 9 and the upstream or downstream motif might also contribute to the loss of Tyr iodination in hTg (see asterisks at given columns in Figure 9 and footnote).

##### 4.7.2. Forming solely DIT in mTg but not iodinated in hTg, and vice versa

The mTyr810/hTyr809 pair has no iodination motif (Figure 10). However, at least a change in any of the five positions of any 5-residue TH-binding motif is detected, and this change might account for the different iodination pattern of this Tyr pair. The change concerns the second position in the downstream perfect motif at columns 22-26. Similarly to the Tyr2617 pair shown in Figure 9 (MIT-forming in hTg, but not iodinated in mTg), the Tyr2697 pair both has an E-X-Y iodination motif, with an E-N-Y version in the DIT-forming hTg but E-S-Y version in the not iodinated mTg. However, this is not the only difference between mTyr2697 and hTyr2697.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 219-297	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	<b>9</b>	<b>S</b>	<b>G</b>	<b>Y235</b>	<b>C</b>	<b>Y</b>	<b>C</b>	<b>A</b>	<b>D</b>	<b>S</b>	<b>Q</b>	<b>G</b>
hTg, 218-296	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	<b>9</b>	<b>S</b>	<b>G</b>	<b>y234</b>	<b>C</b>	<b>H</b>	<b>C</b>	<b>A</b>	<b>D</b>	<b>S</b>	<b>Q</b>	<b>G</b>
mTg, 316-428	<b>Y</b>	<b>I</b>	<b>P</b>	<b>R</b>	<b>C</b>	<b>54</b>	<b>R</b>	<b>F</b>	<b>Y377</b>	<b>F</b>	<b>E</b>	<b>T</b>	<b>P</b>	<b>D</b>	<b>Y</b>	<b>F</b>	<b>S</b>
hTg, 315-428	<b>Y</b>	<b>V</b>	<b>P</b>	<b>S</b>	<b>C</b>	<b>54<sup>1</sup></b>	<b>R</b>	<b>L</b>	<b>y376</b>	<b>F</b>	<b>G</b>	<b>T</b>	<b>S</b>	<b>G<sup>1</sup></b>	<b>Y</b>	<b>F</b>	<b>S</b>
mTg, 1105-1284	<b>F</b>	<b>I</b>	<b>P</b>	<b>V</b>	<b>C</b>	<b>3</b>	<b>G</b>	<b>E</b>	<b>Y1115</b>	<b>V</b>	<b>R</b>	<b>K</b>	<b>Q</b>	<b>T</b>	<b>S</b>	<b>G</b>	<b>T</b>
hTg, 1104-1283	<b>F</b>	<b>V</b>	<b>P</b>	<b>A</b>	<b>C</b>	<b>3</b>	<b>G</b>	<b>E</b>	<b>y1114</b>	<b>A</b>	<b>R</b>	<b>L</b>	<b>Q</b>	<b>A</b>	<b>S</b>	<b>G</b>	<b>A</b>
mTg, 1431-1564	<b>F</b>	<b>V</b>	<b>T</b>	<b>S</b>	<b>P</b>	<b>42</b>	<b>G</b>	<b>T</b>	<b>Y1480</b>	<b>Q</b>	<b>E</b>	<b>Q</b>	<b>A</b>	<b>G</b>	<b>S</b>	<b>S</b>	<b>A</b>
hTg, 1430-1565	<b>F</b>	<b>G</b>	<b>T</b>	<b>S</b>	<b>P</b>	<b>44<sup>1</sup></b>	<b>G</b>	<b>F</b>	<b>y1481</b>	<b>Q</b>	<b>E</b>	<b>Q</b>	<b>A</b>	<b>G</b>	<b>S</b>	<b>L</b>	<b>A</b>
mTg, 1875-1960	<b>W</b>	<b>L</b>	<b>F</b>	<b>T</b>	<b>H</b>	<b>31</b>	<b>S</b>	<b>L</b>	<b>Y1913</b>	<b>F</b>	<b>T</b>	<b>C</b>	<b>F</b>	<b>L</b>	<b>Y</b>	<b>P</b>	<b>E</b>
hTg, 1878-1963	<b>W</b>	<b>L</b>	<b>F</b>	<b>K</b>	<b>H</b>	<b>31<sup>1</sup></b>	<b>S</b>	<b>L</b>	<b>y1916</b>	<b>F</b>	<b>T</b>	<b>C</b>	<b>T</b>	<b>L</b>	<b>Y</b>	<b>P</b>	<b>E</b>
mTg, 2630-2697	<b>Y</b>	<b>A</b>	<b>F</b>	<b>G</b>	<b>L</b>	<b>21</b>	<b>M</b>	<b>Q</b>	<b>Y2658</b>	<b>F</b>	<b>S</b>	<b>N</b>	<b>F</b>	<b>I</b>	<b>R</b>	<b>S</b>	<b>G</b>
hTg, 2630-2686	<b>F</b>	<b>A</b>	<b>L</b>	<b>G</b>	<b>L</b>	<b>21</b>	<b>M</b>	<b>Q<sup>1</sup></b>	<b>y2658</b>	<b>F</b>	<b>S</b>	<b>H</b>	<b>F</b>	<b>I</b>	<b>R</b>	<b>S</b>	<b>G</b>
mTg, 2609-2642	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>V</b>	<b>P</b>	<b>E</b>	<b>S</b>	<b>y2617</b>	<b>G</b>	<b>H</b>	<b>G</b>	<b>S</b>	<b>L</b>	<b>E</b>	<b>L</b>	<b>L</b>
hTg, 2609-2642	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>A</b>	<b>P</b>	<b>E</b>	<b>N</b>	<b>Y2617</b>	<b>G</b>	<b>H</b>	<b>G</b>	<b>S</b>	<b>L</b>	<b>E</b>	<b>L</b>	<b>L</b>
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 219-297	<b>R</b>	<b>E</b>	<b>L</b>	<b>38</b>	<b>F</b>	<b>L</b>	<b>A</b>	<b>I</b>	<b>Q</b>	<b>L</b>	<b>V</b>	<b>I</b>	<b>S</b>	<b>G</b>	<b>R</b>	<b>F</b>	<b>R</b>
hTg, 218-296	<b>R</b>	<b>E</b>	<b>L</b>	<b>38<sup>1</sup></b>	<b>F</b>	<b>L</b>	<b>A</b>	<b>V</b>	<b>Q</b>	<b>S</b>	<b>V</b>	<b>I</b>	<b>S</b>	<b>G</b>	<b>R</b>	<b>F</b>	<b>R<sup>1</sup></b>
mTg, 316-428	<b>P</b>	<b>Q</b>	<b>D</b>	<b>27</b>	<b>F</b>	<b>V</b>	<b>D</b>	<b>S</b>	<b>G</b>	<b>L</b>	<b>L</b>	<b>R</b>	<b>S</b>	<b>I</b>	<b>A</b>	<b>V</b>	<b>E</b>
hTg, 315-428	<b>Q</b>	<b>H</b>	<b>D</b>	<b>28</b>	<b>F</b>	<b>V</b>	<b>D</b>	<b>S</b>	<b>G</b>	<b>L</b>	<b>L</b>	<b>R</b>	<b>P</b>	<b>M</b>	<b>V</b>	<b>E</b>	<b>G</b>
mTg, 1105-1284	<b>G</b>	<b>T</b>	<b>W</b>	<b>145</b>	<b>W</b>	<b>V</b>	<b>T</b>	<b>L</b>	<b>P</b>	<b>P</b>	<b>P</b>	<b>R</b>	<b>A</b>	<b>C</b>	<b>Q</b>	<b>R</b>	<b>P</b>
hTg, 1104-1283	<b>G</b>	<b>T</b>	<b>W</b>	<b>145<sup>1</sup></b>	<b>W</b>	<b>E</b>	<b>S</b>	<b>Q</b>	<b>L</b>	<b>P</b>	<b>Q</b>	<b>P</b>	<b>R</b>	<b>A</b>	<b>C</b>	<b>Q</b>	<b>R</b>
mTg, 1431-1564	<b>C</b>	<b>I</b>	<b>P</b>	<b>60</b>	<b>W</b>	<b>L</b>	<b>Q</b>	<b>T</b>	<b>E</b>	<b>A</b>	<b>G</b>	<b>L</b>	<b>S</b>	<b>E</b>	<b>S</b>	<b>Q</b>	<b>C</b>
hTg, 1430-1565	<b>C</b>	<b>V</b>	<b>P</b>	<b>60<sup>1</sup></b>	<b>W</b>	<b>W</b>	<b>E</b>	<b>T</b>	<b>E</b>	<b>A</b>	<b>P</b>	<b>L</b>	<b>E</b>	<b>D</b>	<b>S</b>	<b>Q</b>	<b>C</b>
mTg, 1875-1960	<b>A</b>	<b>Q</b>	<b>V</b>	<b>23</b>	<b>F</b>	<b>R</b>	<b>R</b>	<b>K</b>	<b>V</b>	<b>V</b>	<b>L</b>	<b>N</b>	<b>D</b>	<b>R</b>	<b>V</b>	<b>K</b>	<b>N</b>
hTg, 1878-1963	<b>A</b>	<b>Q</b>	<b>V</b>	<b>23<sup>1</sup></b>	<b>F</b>	<b>R</b>	<b>K</b>	<b>K</b>	<b>V</b>	<b>I</b>	<b>L</b>	<b>E</b>	<b>D</b>	<b>K</b>	<b>V</b>	<b>K</b>	<b>N</b>
mTg, 2630-2697	<b>N</b>	<b>P</b>	<b>N</b>	<b>15</b>	<b>W</b>	<b>P</b>	<b>D</b>	<b>F</b>	<b>I</b>	<b>P</b>	<b>G</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>E</b>	<b>S</b>	<b>Y</b>
hTg, 2630-2686	<b>N</b>	<b>P</b>	<b>N</b>	<b>4</b>	<b>F</b>	<b>S</b>	<b>R</b>	<b>K</b>	<b>V</b>	<b>P</b>	<b>T</b>	<b>F</b>	<b>A</b>	<b>T</b>	<b>P</b>	<b>W</b>	<b>P</b>
mTg, 2609-2642	<b>A</b>	<b>D</b>	<b>V</b>	<b>Q</b>	<b>Y</b>	<b>A</b>	<b>F</b>	<b>G</b>	<b>L</b>	<b>P</b>	<b>F</b>	<b>Y</b>	<b>S</b>	<b>A</b>	<b>Y</b>	<b>Q</b>	<b>G</b>
hTg, 2609-2642	<b>A</b>	<b>D</b>	<b>V</b>	<b>Q</b>	<b>F</b>	<b>A</b>	<b>L</b>	<b>G</b>	<b>L</b>	<b>P</b>	<b>F</b>	<b>Y</b>	<b>P</b>	<b>A</b>	<b>Y</b>	<b>E</b>	<b>G</b>

**Figure 9.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines that form MIT only in mTg or only in hTg (column 9) and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: A lower case Y (y) in column 9 indicates that such tyrosine is not iodinated. Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>Mutations of hTg residues associated with congenital hypothyroidism: L253, R296, Q329, G381, C1264, W1437, S1466, D1513, R1530, C1897, I1931, Q2657.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg,759-833	<b>Y</b>	<b>I</b>	P	Q	<b>C</b>	44	V	S	Y810	R	E	V	A	S	R	N	<b>F</b>
hTg, 759-832	<b>Y</b>	<b>I</b>	P	Q	<b>C</b>	43	M	S	y809	R	E	A	A	S	G	N	<b>F</b>
mTg, 2688-2730	<b>F</b>	<b>I</b>	P	G	A	2	E	S	y2697	K	E	L	S	A	Q	L	P
hTg, 2688-2730	<b>F</b>	<b>V</b>	P	R	A	2	E	N	Y2697	K	E	<b>F</b>	S	E	L	<b>L</b>	P
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg,759-833	S	L			<b>F</b>	<b>L</b>	Q	S	<b>L</b>	Y	D	A	G	Q	Q	R	I
hTg, 759-832	S	L			<b>F</b>	<b>I</b>	Q	S	<b>L</b>	Y	E	A	G	Q	Q	D	V
mTg, 2688-2730	N	R	Q	9	<b>W</b>	S	K	<b>Y</b>	<b>I</b>	Q	T	<b>L</b>	K	D	A	G	N
hTg, 2688-2730	N	R	Q	9	<b>W</b>	S	K	<b>Y</b>	<b>I</b>	S	S	<b>L</b>	K	T	S	A	D

**Figure 10.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines not iodinated in mTg, but iodinated as DIT in hTg (column 9) and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: A lower case Y (y) in column 9 indicates that such tyrosine is not iodinated. Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**.

Indeed, there are amino acid changes at two positions (second and fourth) in the upstream TH-binding motif and another two (third and fourth position) in the perfect TH-binding motif at columns 25-29.

#### 4.7.3. Forming MIT and DIT only in mTg, but not iodinated in hTg

Of the six such Tyr, only mTyr383 has a iodination consensus motif (D-Y), which is lost in the hTg counterpart (Figure 11). However, at least one change at any of the five positions in any 5-residue TH-binding motif is apparent for five hTyr. In hTyr382, hTyr1165 and hTyr1446 the change occurs in the second position of the upstream motif, and in hTyr2478 in both the third and fifth position. The last position also changes in the motif upstream of hTyr1165. hTyr1005 has no change both in the upstream motif (columns 1-5) and the downstream motif (columns 22-26). However, hTyr1005 (that is, the counterpart of mTyr1006) occupied the second position in the imperfect motif at columns 8-12, a motif that is lost because Leu in the fifth position in mTg is replaced by Arg in hTg. Interestingly, this Arg (R1008) is one of the amino acids mutated in patients with congenital hypothyroidism due to Tg mutations. Other mutations occur at residues more or less close to the six hTyr residues in Figure 11 (see cells with asterisks and footnote). A change in the fifth position (column 14 in Figure 11) of an imperfect motif that follows immediately hTyr in column 9 concerns hTyr2478.

#### 4.7.4. Tyrosines that form both MIT and DIT in mTg, but only MIT in hTg

The mTyr259/hTyr258 pair has the same iodination motif (E-X-Y in the version of E-I-Y), whereas the X residue differs in the mTyr785/hTyr785

pair (Figure 12). Thus, the iodination motif does not provide a consistent explanation for both hTyr258 and hTyr785 losing the DIT-formation property of their mTyr counterparts. There is no difference either in the sequence and spacing from the upstream 5-residue thyroid hormone motif within each pair. However, some changes occur in the downstream motif. Downstream of hTyr258, the fourth position of the motif is occupied by Val (as opposed to Ile in the corresponding position of mTg), while downstream of hTyr785 there is a composite motif that spans columns 22-29 and with an interval a bit longer from hTyr785. This composite motif in mTg consists of an imperfect **FSLFL** motif at columns 22-26 and a perfect **FLQSL** motif at columns 25-29. In hTg, the first motif differs at three positions (second, third and fourth) and the second motif at another three positions (third, fourth and fifth).

#### 4.7.5. Tyrosines that form both MIT and DIT in mTg, but both MIT and T4 in hTg

Unlike the E-X-Y iodination motif of the two MIT- and DIT-forming mTyr in Figure 12 (mTyr258 and mTyr785), the MIT- and DIT-forming mTyr2587 in Figure 13 has the D-Y iodination motif. The same motif is shared by the MIT- and T4-forming hTyr2587. Like the mTyr258/hTyr258 and mTyr785/hTyr785 pairs, where the human component loses DIT-formation, the switch from DIT-formation in mTyr2587 to T4-formation in hTyr2587 cannot be accounted for by changes in the upstream 5-residue TH-binding motif or spacing size (Figure 13). There is no change either in the TH-binding motif that immediately follows Tyr2587 (columns 10-14). Instead, similarly to Y258 and Y785 (Figure 12), at least one change occurs in the downstream motif at columns 22-26. This is a perfect motif, and the change occurs in the fifth position (Figure 13).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 316-428	<b>Y</b>	<b>I</b>	P	R	<b>C</b>	60	P	D	Y383	F	S	P	Q	D	L	L	S
hTg, 315-428	<b>Y</b>	<b>V</b>	P	S	<b>C</b>	60 <sup>1</sup>	S	G <sup>1</sup>	y382	F	S	Q	H	D	L	F	S
mTg, 993-1040	<b>Y</b>	A	I	R	<b>L</b>	6	T	<b>F</b>	Y1006	Q	S	<b>L</b>	R	A	S	L	G
hTg, 992-1039	<b>Y</b>	A	I	R	<b>L</b>	6	S	<b>F</b>	y1005	Q	R	R <sup>1</sup>	R	F	S	P <sup>1</sup>	D
mTg, 1115-1284	<b>Y</b>	<b>V</b>	R	K	Q	44	L	G	Y1166	S	P	V	<b>C</b>	E	A	L	D
hTg, 1114-1283	Y	A	R	L	Q	44	P	G	<b>y1165</b>	<b>V</b>	P	A	<b>C</b>	R	A	E	D
mTg, 1431-1564	<b>F</b>	<b>V</b>	T	S	P	8	G	F	Y1446	R	V	P	T	T	R	Q	D
hTg, 1430-1565	F	G	T	S	P	8 <sup>1</sup>	G	<b>F</b>	y1445	Q	V	<b>L</b>	T	S	E	A	S
mTg, 2407-2499	<b>F</b>	R	K	A	<b>L</b>	64	F	H	<b>Y2478</b>	<b>W</b>	G	P	<b>V</b>	<b>V</b>	D	G	Q
hTg, 2407-2497	<b>F</b>	R	R	A	<b>V</b>	64	F	H	<b>y2478</b>	<b>W</b>	G	P	<b>V</b>	<b>I</b>	D	G	H
mTg, 2662-2700	<b>F</b>	<b>I</b>	R	S	G	N	P	N	Y2670	P	H	E	F	S	R	K	A
hTg, 2662-2700	<b>F</b>	<b>I</b>	R	S	G	N	P	N	y2670	P	Y	E	<b>F</b>	S	R	K	<b>V</b>
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 316-428	S	E	D	21	<b>F</b>	<b>V</b>	D	S	G	L	L	R	S	I	A	V	E
hTg, 315-428	S	P	E	22	<b>F</b>	<b>V</b>	D	S	G	L	L	R	P	M	V	E	G
mTg, 993-1040	K	S	D	10	<b>Y</b>	<b>M</b>	P	Q	<b>C</b>	N	M	I	G	G	W	E	P
hTg, 992-1039	D	S	A	10	<b>Y</b>	<b>M</b>	P	Q	<b>C</b>	D	A	F	G	S	W	E	P
mTg, 1115-1284	G	A	F	94	<b>W</b>	<b>V</b>	T	L	P	P	P	R	A	<b>C</b>	Q	R	P
hTg, 1114-1283	G	G	F	94 <sup>1</sup>	<b>W</b>	E	S	Q	<b>L</b>	P	Q	P	R	A	<b>C</b>	Q	R
mTg, 1431-1564	A	L	G	94	<b>W</b>	<b>L</b>	Q	T	E	A	G	L	S	E	S	Q	<b>C</b>
hTg, 1430-1565	Q	D	G	96 <sup>1</sup>	<b>W</b>	<b>W</b>	E	T	E	A	P	L	E	D	S	Q	<b>C</b>
mTg, 2407-2499					<b>Y</b>	<b>L</b>	R	E	<b>L</b>	P	S	R	R	L	K	R	P
hTg, 2407-2497					<b>F</b>	<b>L</b>	R	E	P	P	A	R	A	L	K	R	S
mTg, 2662-2700	A	E	F	6	<b>F</b>	<b>I</b>	P	G	A	G	G	E	S	Y	K	E	L
hTg, 2662-2700	P	T	F	6	<b>F</b>	<b>V</b>	P	R	A	G	G	E	N	Y	K	E	F

**Figure 11.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mTg as MIT and DIT but not iodinated in hTg (column 9) and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: A lower case Y (y) in column 9 indicates that such tyrosine is not iodinated. Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>Mutations of hTg residues associated with congenital hypothyroidism: Q329, G381, R1008, P1012, C1264, W1437, C1466, D1513, R1530.

#### 4.7.6. Tyrosines that form MIT, DIT and T4 in mTg, but MIT, T4 and T3 in hTg

Here the hTyr (Tyr24) maintains the MIT- and T4-forming property of mTyr25, but switches the DIT-formation to T3-formation, though the iodination motif is the same (E-Y) in the two Tyr (Figure 14). The pair

mTyr25/hTyr24 occupies the second position of an imperfect motif (columns 7-11), which is followed by another two imperfect motifs equally spaced, with none of the three motifs having even a single amino acid change. Instead, the upstream motifs differ. hTyr25 is preceded by the imperfect motif WVSAN (aa 16-20), which is lost in mTg (LVAAN, aa 17-21). However,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 219-297	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	33	<b>E</b>	<b>I</b>	Y259	<b>D</b>	<b>T</b>	<b>I</b>	<b>F</b>	<b>A</b>	<b>G</b>	<b>L</b>	<b>D</b>
hTg, 218-296	<b>F</b>	<b>V</b>	<b>T</b>	<b>F</b>	<b>S</b>	33 <sup>1</sup>	<b>E</b>	<b>I</b>	Y258	<b>D</b>	<b>T</b>	<b>I</b>	<b>F</b>	<b>A</b>	<b>G</b>	<b>L</b>	<b>D</b>
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 219-297	<b>Q</b>	<b>A</b>	<b>S</b>	14	<b>F</b>	<b>L</b>	<b>A</b>	<b>I</b>	<b>Q</b>	<b>L</b>	<b>V</b>	<b>I</b>	<b>S</b>	<b>G</b>	<b>R</b>	<b>F</b>	<b>R</b>
hTg, 218-296	<b>L</b>	<b>P</b>	<b>S</b>	14	<b>F</b>	<b>L</b>	<b>A</b>	<b>V</b>	<b>Q</b>	<b>S</b>	<b>V</b>	<b>I</b>	<b>S</b>	<b>G</b>	<b>R</b>	<b>F</b>	<b>R</b>
mTg, 759-830	<b>D</b>	<b>G</b>	<b>Q</b>	21	<b>F</b>	<b>S</b>	<b>L</b>	<b>F</b>	<b>L</b>	<b>Q</b>	<b>S</b>	<b>L</b>	<b>Y</b>	<b>D</b>	<b>A</b>	<b>G</b>	<b>Q</b>
hTg, 759-829	<b>G</b>	<b>Q</b>	<b>D</b>	20	<b>F</b>	<b>S</b>	<b>L</b>	<b>F</b>	<b>I</b>	<b>Q</b>	<b>S</b>	<b>L</b>	<b>Y</b>	<b>E</b>	<b>A</b>	<b>G</b>	<b>Q</b>

**Figure 12.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mTg as containing only both MIT and DIT but containing only MIT in hTg (column 9), and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>L253fsX256 (L234fsX237 in the mature protein) mutation in hTg.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 2576-2621	<b>F</b>	<b>S</b>	<b>R</b>	<b>A</b>	<b>L</b>	4	<b>R</b>	<b>D</b>	Y2587	<b>F</b>	<b>I</b>	<b>I</b>	<b>C</b>	<b>P</b>	<b>M</b>	<b>V</b>	<b>N</b>
hTg, 2576-2621	<b>F</b>	<b>S</b>	<b>R</b>	<b>A</b>	<b>L</b>	4	<b>R</b>	<b>D</b>	Y2587	<b>F</b>	<b>I</b>	<b>I</b>	<b>C</b>	<b>P</b>	<b>I</b>	<b>I</b>	<b>D</b>
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 2576-2621	<b>M</b>	<b>A</b>	<b>S</b>	10	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>V</b>	<b>P</b>	<b>E</b>	<b>S</b>	<b>Y</b>	<b>G</b>	<b>H</b>	<b>G</b>	<b>S</b>
hTg, 2576-2621	<b>M</b>	<b>A</b>	<b>S</b>	10	<b>F</b>	<b>M</b>	<b>Y</b>	<b>H</b>	<b>A</b>	<b>P</b>	<b>E</b>	<b>N</b>	<b>Y</b>	<b>G</b>	<b>H</b>	<b>G</b>	<b>S</b>

**Figure 13.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mTg as containing both MIT and DIT but containing MIT and T4 in human Tg (column 9), and their closest upstream and downstream TH-binding motifs. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 17-55 <sup>1</sup>	<b>L</b>	<b>V</b>	<b>A</b>	<b>A</b>	<b>N</b>	<b>I</b>	<b>F</b>	<b>E</b>	Y25	<b>Q</b>	<b>V</b>	<b>D</b>	<b>A</b>	<b>Q</b>	<b>P</b>	<b>L</b>	<b>R</b>
hTg, 16-54	<b>W</b>	<b>V</b>	<b>S</b>	<b>A</b>	<b>N</b>	<b>I</b>	<b>F</b>	<b>E</b>	Y24	<b>Q</b>	<b>V</b>	<b>D</b>	<b>A</b>	<b>Q</b>	<b>P</b>	<b>L</b>	<b>R</b>
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 17-55	<b>P</b>	<b>C</b>	<b>E</b>	6	<b>F</b>	<b>L</b>	<b>K</b>	<b>Q</b>	<b>A</b>	<b>E</b>	<b>Y</b>	<b>V</b>	<b>P</b>	<b>Q</b>	<b>C</b>	<b>S</b>	<b>E</b>
hTg, 16-54	<b>P</b>	<b>C</b>	<b>E</b>	6 <sup>2</sup>	<b>F</b>	<b>L</b>	<b>K</b>	<b>Q</b>	<b>A</b>	<b>D</b>	<b>Y</b>	<b>V</b>	<b>P</b>	<b>Q</b>	<b>C</b>	<b>A</b>	<b>E</b>

**Figure 14.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mouse Tg as containing MIT, DIT and T4, but containing MIT, T4 and T3 (viz, maintaining MIT and T4-forming property but exchanging DIT for T3) in human Tg (column 9), and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: Perfect motifs are written on a dark gray background, imperfect motifs on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**. <sup>1</sup>For mTg a perfect upstream motif is **WVSTL** (aa 7-11), corresponding to **FTLLA** (aa 8-12) in hTg. <sup>2</sup>R38K (R19K in the mature protein) mutation of hTg.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 1296-1350	<b>F</b>	Q	L	L	<b>L</b>	7	V	D	<b>Y1310</b>	S	G	L	L	Q	A	<b>F</b>	Q
hTg, 1296-1350	<b>F</b>	Q	L	Q	<b>L</b>	7	A	D	<b>Y1310</b>	A	D	L	L	Q	T	<b>F</b>	Q

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 1296-1350	V	F	<b>I</b>	16	<b>F</b>	G	T	L	<b>V</b>	S	S	T	V	<b>C</b>	D	N	S
hTg, 1296-1350	V	F	<b>I</b>	16	<b>F</b>	G	T	L	<b>V</b>	S	I	P	V	<b>C</b>	N	N	S

**Figure 15.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mouse Tg as containing MIT, DIT, T4 and T3, but containing MIT, T4 and T3 (viz, maintaining MIT, T4 and T3-forming property but losing DIT-forming property) in human Tg (column 9), and their closest upstream and downstream TH-binding motifs. Footnote: Imperfect motifs are written on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. Cysteines are typed in **red**.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 987-1017	<b>F</b>	<b>L</b>	R	G	G			E	<b>Y993</b>	A	I	R	L	A	A	Q	S
hTg, 986-1016	<b>F</b>	<b>L</b>	R	G	S <sup>1</sup>			D	<b>Y992</b>	A	I	R	L	A	A	Q	S

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 987-1017	T	L	T		<b>F</b>	<b>Y</b>	Q	S	<b>L</b>	R	A	S	L	G	K	S	D
hTg, 986-1016	T	L	S		F	Y	Q	R	R	R	F	S	P <sup>2</sup>	D	D	S	A

**Figure 16.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mTg as MIT, DIT and T3, but containing MIT and DIT in hTg, thus losing T3-formation (column 9), and their closest upstream and downstream TH-binding motifs. When a motif is not conserved in either Tg, the amino acids in the corresponding positions are shown, without boldface letters and on white background. Footnote: Imperfect motifs are written on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. <sup>1</sup>S990I (S971I in the mature protein) mutation in hTg. <sup>2</sup>P1012L (P993L in the mature protein) mutation in hTg.

mTg has a more N-terminal, perfect motif (**WVSTL**, aa 7-11) that is lost in the corresponding sequence of hTg (FTLLA, aa 8-12).

#### 4.7.7. Tyrosines that form MIT, DIT, T4 and T3 in mTg, but MIT, T4 and T3 in hTg

There is only one such Tyr, Y1310 in both species (Figure 15). Here, the hTg counterpart loses only the DIT-formation. Again, the iodination motif cannot explain the difference in the iodination pattern between mTg and hTg, because it is of the type D-Y in both (Figure 15). Similarly to the mTyr25/hTyr24 pair (which shares with the mTyr1310/hTyr1310 pair the maintainment of MIT and T4-formation but loses DIT-formation in hTg), there is some change only in the upstream motif. Such change occurs in the fourth position, not in the downstream motifs (Figure 15). However, hTyr1310 occupies the first position of imperfect motif, with the second and third position occupied by amino acids different from those in mTg.

#### 4.7.8. Tyrosines that form MIT, DIT and T3 in mTg, but only MIT and DIT in hTg

Here, the hTyr counterpart loses T3 formation. Unlike the MIT-, DIT- and T3-forming mTyr1310 (which has a D-Y iodination motif; Figure 15), mTyr993 has the E-Y iodination motif. Also, a D-Y iodination motif is

featured by hTyr992, which does not form T3 (Figure 16). Both the upstream and downstream 5-residue TH-binding motif have an amino acid change (fifth, and fourth and fifth position, respectively). Noteworthy, the change in the fifth position causes hTyr992 to lose the downstream motif (Figure 16) and, as a probable consequence, hTyr992 fails to form T3.

#### 4.7.9. Tyrosines that form only MIT in mTg, but only DIT in hTg

Here, mTyr884 has no iodination motif, whereas the mPro883 to hSer882 change causes hTyr883 to acquire the S-Y-S iodination motif (Figure 17). However, this is not the sole difference. Indeed, there are two changes (second and third position) in the first TH-binding motif downstream and one change (third position) in the more downstream TH-binding motif.

#### 4.7.10. Summary

There are 37 iodinated Tyr in mTg, but only 16 in hTg (11). Only 16/37 mTyr (43%), as opposed to 15/16 hTyr (94%,  $P = 0.0006$  by Fisher's exact test), have any of the known iodination motifs. However, there are instances in Tg of either species where possession of the iodination motif does not ensure Tyr iodination. Furthermore, the same iodination motif is not associated with the same panel of formed



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
mTg, 869-948	<b>F</b>	<b>W</b>	<b>Q</b>	<b>I</b>	<b>L</b>	8	G	P	Y884	S	D	<b>F</b>	<b>N</b>	<b>M</b>	<b>P</b>	<b>L</b>	E
hTg, 868-947	<b>F</b>	<b>W</b>	Q <sup>1</sup>	<b>I</b>	<b>L</b>	8	G	S	Y883	S	D	<b>F</b>	S	T	P	<b>L</b>	A

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
mTg, 869-948	H	F	N	40	<b>F</b>	<b>I</b>	K	E	T	E	E	I	V	S	A	S	N
hTg, 868-947	H	F	D	40	<b>F</b>	<b>I</b>	R	E	T	E	E	I	V	S	A	S	N

**Figure 17.** Comparison between the amino acid sequences of mouse Tg (mTg) and human Tg (hTg) which include tyrosines iodinated in mTg as only MIT, but as only DIT in hTg (column 9), and their closest upstream and downstream TH-binding motifs. Footnote: Imperfect motifs are written on a light gray background. Residues that match those at first, second or fifth position of the 5-residue motif (F/Y/W, I/L/M/V, X, X, I/L/M/V) are typed **boldface**. <sup>1</sup>Q870H (Q851H in the mature protein) mutation in hTg.

iodinated tyrosines and/or thyronines, as resulting from both intraspecies and interspecies analyses. To complete the picture of inconsistencies, the same panel of formed iodinated tyrosines and/or thyronines is associated with different iodination motifs.

In contrast, our detection of perfect or imperfect versions of a 5-residue TH-binding motif (Y, L/I/M, X, X, V/L/I) that was previously found to be conserved in TH-binding plasma proteins (2) and updated into Y/F/W, L/I/V/M, X, X, L/I/V/M (3) or W/F/Y, L/I/V/M, I/V/M/L, P, L/V/I/M upon considering also cell-associated TH-interacting proteins (*viz.* membrane transporters, selenodeiodinases and TH nuclear receptors) (4) provides more consistency. First, the iodinated Tyr of either species is always preceded and followed by at least one such motif, and sometimes the iodinated Tyr occupies the first position of the motif. Second, the aforementioned inconsistencies in the iodination/not iodination property of Tyr and/or panel of iodination products find always an explanation in at least one of the TH-binding motifs flanking or containing the Tyr in question. Third, a number of residues of hTg that are mutated in patients with congenital forms of hypothyroidism occupy one of the five positions of the TH-binding motif or are very close to it.

## 5. DISCUSSION

As summarized at the end of the Results, this *in silico* research represents an effort in trying to understand why only a limited number of tyrosines in Tg are iodinated and why, of these, some form one or both TH precursors (MIT, DIT) while others form one or both TH (T3, T4). Explanations cannot be sorted out by the simple possession and sequence of any of the classical iodination motifs. Because in practical terms the synthesis of either TH precursors or TH within a protein is equivalent to binding of iodotyrosines/iodothyronines to a circulating or cellular protein –based on the fact that the iodothyronines/iodotyrosines need to occupy an optimal 3-dimensional local site–, we reasoned (see Introduction) that this

optimal local environment could have been provided by a phylogenetically conserved 5-residue TH-binding motif, a motif which was originally found in a few TH plasma carriers. Thus, the presence of this 5-residue TH-binding motif might have ensured a spatial environment optimal for occupancy by any of MIT, DIT, T3 and T4 in Tg.

The consensus motif resulting from 426 sequences of thyroid hormone plasma transport proteins (*Homo sapiens* and 94 animal species) was Y/F/W, L/V/I/M, L/V/M/I, L/V/I/M, V/L/I/M. The consensus motif resulting from 8691 sequences (*Homo sapiens* and 368 animal species) of cell-membrane TH transporters was W/F/Y, L/I/V/M, I/V/L/M, P, L/V/I/M. The consensus motif resulting from 624 sequences of TH nuclear receptors (*Homo sapiens* and 209 animal species) was F/W/Y, W, P, K, L. The consensus motif from 488 sequences of the selenodeiodinases stands out in that the motif is imperfect, with an amino acid other than I/L/V/M in the fifth position. The TH-binding motif in the deiodinases is F, L/V/M, L/I, V, Y. Noteworthy, the presence of Pro in the third or fourth position of the 5-residue motif, thus matching TH nuclear receptors or cell-membrane transporters, is also present in mTg (YLPQC; FMPVQ; YIPRC; FVPSC; FVPTC; FLPVQ; YIPQC; FNMLP; YMPQC; FIPVC; FLYPE; WGPVV; FIPGA) and hTg (YLPQC; FMPVQ; YVPSC; FLPVQ; YIPQC; FSTPL; YMPQC; FVPAC; YVPAC; WGPVI; FVPRA). Pro in the third position of the motif is also present in apoC-II (consensus of 14 sequences), and in the fourth position of TBG, alpha1-anti-chymotrypsin and cortisol-binding protein (consensus of 14, 14 and 7 sequences).

In an *ad hoc* section at the end of the Results, we have summarized certain reasons that provide some consistency to our results. One such consistency is functional in nature, *viz.* a number of residues of hTg that are mutated in patients with congenital forms of hypothyroidism occupy one of the five positions of the TH-binding motif or are very close to it.

In summary, we have provided novel bioinformatics data that have the potential to explain differences within species and between species that cannot be explained by the presence/absence or type of known tyrosine iodination motifs. Such differences concern (i) the iodination or non-deiodination of the same tyrosine in different species; (ii) Tyr iodination in spite of the absence of Tyr iodination motifs; (iii) Tyr non-deiodination in spite of the presence of Tyr iodination motifs; (iv) different patterns of iodotyrosines and/or iodothyronines formed in spite of the same tyrosine iodination motif; (v) same or very similar patterns of iodotyrosines and/or iodothyronines formed in spite of different tyrosine iodination motifs. The presence of perfect/imperfect copies of this motif, which is evolutionarily conserved, in cell proteins that transport or simply bind iodide, iodotyrosines and iodothyronines suggests that an ancestral motif served multiple functions in thyroid physiology. These functions start with iodide uptake into the thyrocyte (13) and subsequent utilization for thyroid hormone synthesis within the protein substrate represented by Tg (the present paper), continue with thyroid hormone transport in blood (3) and thyroid hormone movement across the plasma membrane, and end with thyroid hormone intracellular metabolism by deiodinases and thyroid hormone interaction with the nuclear receptors (3). This represents a parsimonious usage of a motif for multipurpose tasks, a motif that can be modulated with “variations on the theme” based on the specific functional task.

## 6. REFERENCES

1. S. Benvenaga, H.J. Cahnmann, D. Rader, M. Kindt, A. Facchiano, J. Robbins: Thyroid hormone binding to isolated human apolipoproteins A-II, C-I, C-II, and C-III: homology in thyroxine binding sites. *Thyroid* 4, 261-267 (1994)  
DOI: 10.1089/thy.1994.4.261
2. S. Benvenaga: A thyroid hormone binding motif is evolutionarily conserved in apolipoproteins. *Thyroid* 7, 605-611 (1997)  
DOI: 10.1089/thy.1997.7.605
3. S. Benvenaga, F. Guarneri: Conservation in the phylum of the local homology of apolipoproteins with the thyroid hormone plasma carriers. *Rev Endocr Metab Disord* 17, 537-544 (2016)  
DOI: 10.1007/s11154-016-9379-7
4. S. Benvenaga, F. Guarneri: Deiodinases share an evolutionarily conserved thyroid hormone-binding motif. *Front Biosci Landmark Ed* 23, 2195-2204 (2018)
5. J. Bismuth, M. Castay, S. Lissitzky: Binding of 3,5-diiodotyrosine to serum proteins. *Clin Chim Acta* 69, 417-422 (1976)
6. N. Okabe, N. Manabe, R. Tokuoka, K. Tomita: Interaction of diiodo-L-tyrosine and triiodophenol with bovine serum albumin. Circular dichroism and fluorescence studies. *J Biochem* 80, 455-461 (1976)
7. K. Grygorian, H. Shilajyan: Physicochemical peculiarities of iodine-dimethylsulfoxide-H<sub>2</sub>O solutions and effect on ion binding to bovine serum albumin. *Dataset Papers in Chemistry* 2013, Article ID 534328 (2013)  
DOI: 10.7167/2013/534328
8. N.M. Bourgeois, S.L. Van Herck, P. Vancamp, J. Delbaere, C. Zevenbergen, S. Kersseboom, V.M. Darras, T.J. Visser: Characterization of chicken thyroid hormone transporters. *Endocrinology* 157, 2560-2574 (2016)  
DOI: 10.1210/en.2015-2025
9. B.B. Mughal, M. Leemans, E.C. Lima de Souza, S. le Mevel, P. Spirhanzlova, T. Visser, J.B. Fini, B.A. Demeneix: Functional characterisation of Xenopus thyroid hormone transporters McT8 and Oatp1c1. *Endocrinology* 158, 2694-2705 (2017)  
DOI: 10.1210/en.2017-00108
10. B. Di Jeso, P. Arvan: Thyroglobulin from molecular and cellular biology to clinical endocrinology. *Endocr Rev* 37, 2-36 (2016)  
DOI: 10.1210/er.2015-1090
11. A. Dedieu, J.C. Gaillard, T. Pourcher, E. Darrouzet, J. Armengaud: Revisiting iodination sites in thyroglobulin with an organ-oriented shotgun strategy. *J Biol Chem* 286, 259-269 (2011)  
DOI: 10.1074/jbc.M110.159483
12. F. Guarneri, B. Guarneri: Bioinformatic analysis of HLA-linked genetic susceptibility to immunoallergic diseases: the MotiFinder software. *Ann Ital Dermatol Allergol* 64, 69-75 (2010)
13. S. Benvenaga, F. Guarneri: Thyroid hormone-interacting cell and plasma proteins share a common motif. *Front Biosci Schol Ed* 10, 326-336 (2018)  
DOI: 10.2741/S519
14. S. Benvenaga, F. Guarneri: Homology of pendrin, sodium-iodide symporter and apical

iodide transporter. *Front Biosci Landmark Ed* 23, 1864-1873 (2018).  
DOI: 10.2741/4677

15. H.M. Targovnik, C.E. Citterio, C.M. Rivolta: Thyroglobulin gene mutations in congenital hypothyroidism. *Horm Res Paediatr* 75, 311-321 (2011)  
DOI: 10.1159/000324882

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**Send correspondence to:** Fabrizio Guarneri, Department of Clinical and Experimental Medicine - Dermatology, via Consolare Valeria - Gazzi, 98125 Messina, Italy, Tel: +39 090 2212890, Fax: +39 090 2927691, E-mail: f.guarneri@tiscali.it