Enhancing T3 and cAMP responsive gene participation in the thermogenic regulation of fuel oxidation pathways

Ampliando a participação dos genes responsivos a T3 e cAMP na regulação da termogênese gerada pelas vias de oxidação biológica

Karina Kores Dorsa[1], Michelle Venâncio dos Santos[1], Magnus R. Dias da Silva[1]

ABSTRACT

Objective: We sought to identify glycolysis, glycogenolysis, lipolysis, Krebs cycle, respiratory chain, and oxidative phosphorylation enzymes simultaneously regulated by T3 and cAMP. **Materials and methods:** We performed *in silico* analysis of 56 promoters to search for *cis*-cAMP (CREB) and *cis*-thyroid (TRE) response elements, considering UCP1, SERCA2 and glyceraldehyde 3-phosphate dehydrogenase as reference. Only regulatory regions with prior *in vitro* validation were selected. **Results:** 29/56 enzymes presented potential TREs in their regulatory sequence, and some scored over 0.80 (better predictive value 1): citrate synthase, phosphoglucose isomerase, succinate dehydrogenases A/C, UCP3, UCP2, UCP4, UCP5, phosphoglycerate mutase, glyceraldehyde 3-P dehydrogenase, glucokinase, malate dehydrogenase, acyl-CoA transferase (thiolase), cytochrome a3, and lactate dehydrogenase. Moreover, some enzymes have not yet been described in the literature as genomically regulated by T3. **Conclusion:** Our results point to other enzymes which may possibly be regulated by T3 and CREB, and speculate their joint roles in contributing to the optimal thermogenic acclimation. Arq Bras Endocrinol Metab. 2010;54(4):381-9

Keywords
Thermogenesis; T3R; CREB; genomic regulation; fuel oxidation

[1] Universidade Federal de São Paulo (Unifesp), São Paulo, SP, Brazil

RESUMO

Objetivo: Identificar enzimas das vias da glicólise, glicogenólise, lipólise, ciclo de Krebs, cadeia respiratória e fosforilação oxidativa possivelmente reguladas por T3 e cAMP. **Materiais e métodos:** Analisamos 56 genes metabólicos *in silico* mediante a identificação dos elementos *cis* de regulação gênica responsivos ao T3 e cAMP (TREs, *thyroid response elements* e CREs, *cAMP response elements*), utilizando como referência o promotor da UCP1, SERCA2 e gliceraldeído 3-fosfato desidrogenase. Selecionamos somente os promotores com estudo funcional prévio *in vitro*. **Resultados:** 29/56 enzimas apresentaram TREs em suas regiões regulatórias, parte com escore ≥ 0,80 (melhor valor preditivo 1): citrato sintase, fosfoglucose isomerase, succinato desidrogenase A/C, UCP3, UCP2, UCP4, UCP5, fosfoglicerato mutase, gliceraldeído 3-P desidrogenase, glucoquinase, malato desidrogenase, acil-CoA transferase (tiolase), citocromo a3, e lactato desidrogenase; parte desconhecida como regulada por T3. **Conclusão:** Os resultados do presente estudo apontam para novos genes regulados por T3 e cAMP e, por conseguinte, sua contribuição na regulação da termogênese. Arq Bras Endocrinol Metab. 2010;54(4):381-9

Descritores
Termogênese; T3R; CREB; regulação gênica; oxidação biológica

Correspondence to:
Magnus R. Dias da Silva
Laboratório de Endocrinologia Molecular,
Departamento de Bioquímica,
Universidade Federal de São Paulo
04044-020 – São Paulo, SP, Brazil
magnus.bioq@epm.br

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INTRODUCTION

Body heat is a by-product of the transformation of chemical energy slowly released during food oxidation. This apparent thermodynamic inefficiency keeps our body warm and optimizes the functioning of cells and systems. It is widely known that T3 is one of the key

mediators of thermogenesis (1,2), especially through the control of transcriptional uncoupling proteins (UCPs) together with the partner cAMP-CREB, cyclic AMP response element binding protein (1). In fact, UCPs optimize the inefficiency of mitochondrial thermogenesis by shifting the gradient of protons from the ATP synthesis to generate heat (3), as the consequence of proton leak amplification. Despite of the recognized role of T3 in generating heat through UCP canonical thermogenic path (1,4,5), little is known about its transcriptional regulation in other metabolic oxidative enzymes. Much less is clear about T3R-CREB transcriptional regulation partnership in orchestrating the fuel oxidation pathways.

Besides the accumulated evidence on T3R-CREB/UCP thermogenic path (6), we should also consider adding the role of 3,5-diiodo-l-thyronine (T2) and the substrate, or futile cycling contributions to heat generation. In fact, it has been demonstrated that interplay between opposite metabolic routes such as glucose and glucose-6-phosphate and between fructose-6-phosphate and fructose-1,6-diphosphate occurs in man and is directly affected by T3, these substrate cycles contribution may represent a mechanism by which thyroid hormone alters the sensitivity of certain reactions to metabolic signals (7). Moreover, not only T3 but also T2 enhances skeletal muscle mitochondrial thermogenesis by activating pathways involved in the dissipation of the proton-motive force (ATP generation) towards proton leak (heat), the effect being dependent on the presence of free fatty acids inside mitochondria (8). It may also be worth considering further comprehensive overviews on regulation of thermogenesis (1,9,10).

Why search for *cis*-elements in a set of regulatory genomic sequences? In the last decade a great amount of differentially expressed genes was made available by the widespread use of gene expression profiling, especially from microarray and SAGE analysis, without scrutinizing information about their transcriptional regulation. In fact, these methods only take into account arrays created from cDNA sequences of pooled genes, and most of the transcriptional regulatory signals; however, they are more likely to be in the promoter and enhancer/silencer regions.

Although it has been an arduous task delineating the regulatory region of a gene due to the difficulty in establishing the start and the end of its sequence, promoter sequences corresponding to selected cDNAs can be traced either by exon-mapping or discovered by *in silico* promoter prediction tools from whole genome sequences available on public databases (11-13), making it more straightforward. The lack of reliable definition of the promoter size has been overcome with new software, but variability in the limits of regulatory regions still remains. Some programs in bioinformatics, for instance, also perform comparative promoter analysis among co-expressed genes, and then identify common transcription regulatory signals (14-16). These smart web-based tools can be applied to help narrow down an enormous amount of expressed genes provided by selecting canonical transcription factors as filters, in addition to sorting them by specific tissues or pathways.

One of the most important transcriptional factors related to thermogenesis is CREB, which is constitutively expressed in the cell. In fact, CREB activity is greatly increased after phosphorylation by cAMP, which works as a cell sensor of a variety of stimuli, including those downstream of adenilate cyclase activation in the cell membrane (17,18). In addition, the TSH receptor is also mainly coupled to the cAMP cascade, in which the cAMP pathway exerts a dual function as it regulates both thyroid hormone production and proliferation of thyroid epithelial cells (19). Interestingly, we have found that several thermometabolic steps can be simultaneously regulated at the transcriptional level by cAMP-CREB and T3/T3R (20-23), in which we postulate whether both factors do really have an intimate transcriptional crosstalk in generating heat.

MATERIALS AND METHODS

We sought to identify enzymes involved in glycolysis, glycogenolysis, lipid ß-oxidation, Krebs cycle, respiratory chain, and oxidative phosphorylation possibly regulated by T3 and cAMP. We performed *in silico* analysis of 56 promoters related to all enzymes involved in the above-mentioned metabolic routes to search for *cis*-thyroid response elements (TRE) together with c-AMP response elements (CRE), initially using the GENOMATIX program (16), available to academic institutions through the website: http://www.genomatix.de/cgi-bi. The latter predicts the best regulatory sequence of a gene based on the degree of conservation between orthologs, classifying it in the gold, silver or bronze groups accordingly with the best evidence for experimentally verified 5' complete transcript, with 5' end confirmed by the software prediction only, and annotated transcript without confirmation for 5' sequence completeness, respectively.

We have accepted the lowest cutoff threshold obtained from the analysis of the UCP1, SERCA2 and glyceraldehyde 3-phosphate dehydrogenase regulatory regions as reference, since they have been classically demonstrated in the literature as the strongest T3-regulated genes. Indeed, we have only selected regulatory regions with a prior *in vitro* validation of the promoter, according to evidence made available from the GENOMATIX program termed golden promoter.

Once selected, the promoters were followed with a narrowing second screening using three independent programs PROMO (24), ALIBABA2 (25), and TESS (15), all publicly available through sites: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo, http://www.gene-regulation.com/pub/programs/alibaba2, and http://www.cbil.upenn.edu/cgi-bin/tess. TESS uses human CREB (T00163) or T3R-beta1 (T00851) as core position factor filters for TRANSFAC strings, accepting 10% of Maximum Allowable String Mismatch (t_{mm}), a real number ≥ 0 of log-likelihood ratio score (t_{s-a}), and the Minimum string length (t_w) of 6. In fact, most of the available programs for predicting transcription factor binding sites use known sites collected from TRANSFAC major matrix, which contains data on transcription factors, their experimentally-proven binding sites, and an array of regulated genes. Its broad compilation of binding sites allows the derivation of positional weight matrices, so it calculates a p-value for each prediction (26).

These programs are able to specifically search for T3R-b *cis*-element binding sites among all the transcriptional factors on a matrix, grouped within either RXR or CREB family on query regulatory sequences. We have only selected the binding sites for RXRF and CREB that have scored over the defined threshold, assuming a statistically significant p-value < 0.01 (provided by the program).

RESULTS

We were able to identify 29 promoters that presented TREs among all 56 transcriptionally-inspected metabolic enzymes as summarized on table 1. The top 10 enzyme promoters that scored over 0.80 (better predictive value 1) were: citrate synthase, phosphoglucose isomerase, succinate dehydrogenase A/C, UCP3, UCP2, UCP4, UCP5, phosphoglycerate mutase, glycerol 3-phosphate dehydrogenase, glucokinase, malate dehydrogenase, acyl-CoA transferase (thiolase), cytochrome a3, and lactate dehydrogenase. To the best of our knowledge some pulled enzymes have not yet been described in the literature as possibly regulated by T3. Therefore, our results point to more enzymes genomically regulated by T3, possibly broadening its role in the metabolic process of thermogenesis. See table 1 for more details regarding enzyme metabolic pathway and cell localization, their NCBI gene identification, the size of the promoter used for studying, the Initial screening by GENOMATIX program when the score was set on p < 0.01, the second screening using a more specific filter for CRE and TRE by PROMO programs of bioinformatics.

DISCUSSION

Our study extends the participation of T3 regulation to many metabolic enzymes, adding to the understanding of fuel oxidation enzymatic steps for generating heat, as summarized on figure 1. We also demonstrate the usefulness of several *in silico* available resources in searching for potential promoters and for better fishing of transcriptional signals. By using these bioinformatics resources we were able to not only filter for specific transcriptional family factors, but also to compare promoters for common ones. Part of the usefulness of the work described here lies in the ability of rapid screening of gene expression driven by hormonal control. As a result, we observed marked overlapping signals between T3R and CREB (*trans*-elements) signals, in searching for their *cis*-elements (TRE and CRE). From a theoretical viewpoint, we speculate that these transcriptional factors play adjunct roles in orchestrating some oxidative enzymes in the same thermometabolic routes.

Most enzymes regulated by T3R/CREB identified in our study are well known as key steps of fuel metabolism. However, some of them have not yet been described in the literature as being regulated by T3, of which the following stand out: Phosphoglucose Isomerase (Figure 1.2/1.3), Succinate Dehydrogenase C and Succinate Dehydrogenase A (Figure 1.6), NDUFV3/V1/V2 Flavoprotein NADH Dehydrogenase 1 and COX4I1 – Cytochrome a / a3 (Figure 1.7/1.8), Uncoupling Protein 5 (Figure 1.7/1.9), Phosphoglucomutase (Figure 1.3), ACAT1 acetyl-Coenzyme A acetyltransferase 1 (Thiolase 1) and ACAT2 acetyl-Coenzyme A acetyltransferase 2 (Thiolase 2) (Figure 1.1/1.5). These verified enzymes are particularly discussed as follows.

Table 1. *In silico* screening of TRE (RXRF/T3R-b) and CRE (CREB/cAMP) *cis*-regulatory elements of metabolic enzyme

Nº	Metabolic pathway enzyme	Cell localization	NCBI gene ID	Initial screening by GENOMATIX software					Second screening – filtering for T3R-b *cis* specific sites		
				Promoter size (bp)	Total Nº of CREB *cis* sites	CREB (cAMP) score, p < 0.01	Total Nº of RXRF *cis* sites	RXRF (T3R-b) score, p < 0.01	Total Nº PROMO software	Total Nº ALIBABA2 software	Total Nº TESS software
	Glycolysis										
1	Hexokinase	Cytosolic	3098	602	2	2sI - 0.919 / 0.915	3	2sI - 0.853 / 0.816	3	1	0
2	Glucokinase	Cytosolic	2646	668	0	-	1	1sI - 0.859	2	1	0
3	Phosphoglucose Isomerase	Cytosolic	2821	601	4	3sI - 0.771 / 0.912 / 0.944	2	2sI - 0.906 / 0.8	3	2	0
4	Phosphofructokinase 1	Cytosolic	5211	669	1	1sI - 0.888	1	-	0	1	1
5	Aldolase A	Cytosolic	226	668	0	-	0	-	3	0	0
6	Triosephosphate Isomerase	Cytosolic	7167	911	0	-	3	3sI - 0.771 / 0.806 / 0.904	0	1	0
7	Gluceraldehyde 3 Phosphate Dehydrogenase	Cytosolic	26330	718	13	10sI - 0.968 / 0.98 / 1.0 / 1.0 / 0.974 / 0.931 / 0.935 / 0.918 / 0.722 / 0.891	4	3sI - 0.864 / 0.816 / 0.76	4	0	1
8	Phosphoglyrate Kinase	Cytosolic	5232	601	2	1sI - 0.86	5	3sI - 0.76 / 0.769 / 0.791	3	1	0
9	Phosphoglyrate Mutase	Cytosolic	5224	799	1	-	3	3sI - 0.795 / 0.807 / 0.869	2	0	0
10	Enolase	Cytosolic	2027	612	4	4sI - 0.934 / 0.968 / 0.926 / 0.917	4	4sI - 0.796 / 0.793 / 0.77 / 0.786	4	2	0
11	Pyruvate Kinase	Cytosolic	5313	648	3	2sI - 0.846 / 0.875	2	-	2	0	0
12	Lactate Dehydrogenase	Cytosolic	3939	824	9	9sI - 0.909 / 0.97 / 1.0 / 1.0 / 0.976 / 0.888 / 0.894 / .0911 / 0.817	2	2sI - 0.842 / 0.887	1	0	1
	Krebs Cycle										
13	Pyruvate Dehydrogenase (lipoamide) Alpha 1	MM	5160	777	4	3sI - 0.848 / 0.932 / 1.0	1	1sI - 0.847	1	1	2
14	Aconitase	MM	48	663	0	-	3	2sI - 0.81 / 0.872	0	1	0
15	Isocitrate Dehydrogenase	MM	3420	673	2	-	1	-	1	2	0
16	Oxoglutarate (alpha-ketoglutarate) Dehydrogenase	MM	4967	601	11	11sI - 0.854 / 1.0 / 1.0 / 0.945 / 0.962 / 0.88 / 0.9 / 0.926 / 0.921 / 0.892 / 0.908	2	2sI - 0.774 / 0.705	0	1	10
17	Succinyl-CoA Synthetase Subunit Alpha	MM	8802	602	0	-	2	1sI - 0.956	1	0	0
18	Succinate Dehydrogenase C	MM	6391	614	5	4sI - 0.906 / 0.922 / 0.926 / 0.9	0	-	1	2	1
19	SDHA Succinate Dehydrogenase A	MM	6389	611	1	1sI - 0.844	2	2sI - 0.771 / 0.802	1	1	0
20	Succinate Dehydrogenase D	MM	6392	650	0	-	4	4sI - 0.831 / 0.877 / 0.808 / 0.752	2	0	0
21	Fumarate Hydratase	MM	2271	601	2	2sI - 0.879 / 0.908	0	-	2	0	1
22	Malate Dehydrogenase	MM	4190	1141	0	-	5	5sI - 0.854 / 0.75 / 0.799 / 0.803 / 0.809	3	1	2
	Respiratory Chain										
23	NDUFV3 flavoprotein NADH dehydrogenase	Complex I (MMI)	4731	670	2	2sI - 0.976 / 0.998	2	1sI - 0.799	3	0	2
24	NDUFV1 flavoprotein NADH dehydrogenase	Complex I (MMI)	4723	754	4	2sI - 0.941 / 0.983	3	2sI - 0.894 / 0.814	3	2	5
25	NDUFV2 flavoprotein NADH dehydrogenase	Complex I (MMI)	4729	668	4	2sI - 0.918 / 0.926	2	2sI - 0.802 / 0.804	3	0	1
	SDHA flavoprotein succinate dehydrogenase	Complex II (MMI)	6389	611	1	1sI - 0.844	2	2sI - 0.771 / 0.802	1	1	0
26	GPD1 (flavoprotein) glycerol 3-phosphate dehydrogenase (soluble)	External MMI	2819	611	1	1sI - 0.758	2	1sI - 0.761	1	0	2
27	GPD2 (flavoprotein) glycerol 3-phosphate dehydrogenase (mitochondrial)	External MMI	2820	912	4	3sI - 0.888 / 0.834 / 0.906	3	3sI - 0.725 / 0.762 / 0.936	2	3	2
28	CYBA cytochrome b-245, alpha polypeptide	Complex III (MMI)	1535	662	2	2sI - 0.992 / 0.908	2	1sI - 0.779	2	0	2
29	UCRC ubiquinol-cytochrome c reductase complex	Complex III (MMI)	29796	630	1	1sI - 0.888	1	1sI - 0.903	2	0	0

Table 1. Continuation

Nº	Metabolic pathway enzyme	Cell localization	NCBI gene ID	Initial screening by GENOMATIX software					Second screening – filtering for T3R-b *cis* specific sites		
				Promoter size (bp)	Total Nº of CREB *cis* sites	CREB (cAMP) score, p < 0.01	Total Nº of RXRF *cis* sites	RXRF (T3R-b) score, p < 0.01	Total Nº PROMO software	Total Nº ALIBABA2 software	Total Nº TESS software
30	COX4I1 - cytochrome a / a3 subunit IV isoform 1	Complex IV (MMI)	1327	786	1	1sl - 0.921	1	1sl - 0.793	4	1	0
31	SLC25A31 solute carrier family 25, adenine nucleotide translocator member 31	MMI	83447	1049	9	6sl - 0.959 / 0.935 / 0.911 / 0.917 / 0.914 / 0.895	5	3sl - 0.765 / 0.762 / 0.762	3	0	9
32	SLC25A6 solute carrier family 25, adenine nucleotide translocator member 6	MMI	293	772	4	3sl - 0.825 / 0.88 / 0.727	3	2sl - 0.898 / 0.828	0	3	2
33	SLC 25A4 solute carrier family 25, adenine nucleotide translocator member 4	MMI	291	740	0	-	2	1sl - 0.881	4	1	0
34	GPD1 glycerol 3-phosphate dehydrogenase 1 (soluble)	Cytosolic	2819	611	1	1sl - 0.758	2	1sl - 0.761	1	0	2
35	MDH1 malate dehydrogenase 1	MMI (LMA)	4190	1141	0	-	5	5sl - 0.854 / 0.75 / 0.799 / 0.803 / 0.809	6	1	2
36	GOT2 mitochondrial aspartate aminotransferase 2	MMI (LMA)	2806	734	7	5sl - 0.968 / 0.949 / 0.993 / 0.911 / 0.896	4	2sl - 0.804 / 0.769	1	2	4
37	GOT1 aspartate transferase 2	Cytosolic	2805	736	2	1sl - 0.885	1	1sl - 0.818	3	1	0
38	CS citrate synthase	MMI	1431	753	5	2sl - 0.886 / 0.823	6	5sl - 0.872 / 0.823 / 0.847 / 0.971 / 0.888	2	2	6
39	ACLY citrate lyase	Cytosolic	47	749	0	-	0	-	1	0	2
40	UCP1 protein uncoupling 1	MMI (MBF)	7350	726	2	2sl - 0.911 / 0.881	5	4sl - 0.804 / 0.804 / 0.76 / 0.905	4	2	3
41	UCP2 protein uncoupling 2	rather than MBF	7351	1065	4	1sl - 0.901	4	3sl - 0.871 / 0.81 / 0.886	1	0	8
42	UCP3 protein uncoupling 3	rather than MBF	7352	1137	3	3sl - 0.831 / 0.823 / 0.723	6	5sl - 0.874 / 0.804 / 0.88 / 0.874 / 0.791	7	3	1
43	UCP4 protein uncoupling 4	rather than MBF	9481	602	1	1sl - 0.876	4	3sl - 0.862 / 0.845 / 0.8	2	0	1
44	UCP5 protein uncoupling 5	rather than MBF	9016	1183	3	3sl - 0.934 / 0.91 / 0.918	4	3sl - 0.778 / 0.809 / 0.82	6	3	2
	Glycogenolysis										
45	Glycogen phosphorylase	Cytosolic	5834	964	6	3sl - 0.925 / 0.918 / 0.915	2	2sl - 0.809 / 0.818	2	0	0
46	Phosphoglucomutase	Cytosolic	5239	1074	3	2sl - 0.961 / 0.93	2	1sl - 0.798	3	1	0
47	Glucose - 6 - Phosphate Translocase	Cytosolic	2542	601	1	1sl - 0.743	2	1sl - 0.83	2	0	0
48	Glucose - 6 - Phosphatase	Cytosolic	57818	744	2	1sl - 0.878	1	-	1	1	0
	β-Oxidation										
49	Acyl CoA Synthetase (ACSL 6)	Cytosolic	23305	775	2	2sl - 0.937 / 0.914	4	3sl - 0.862 / 0.91 / 0.729	1	1	0
50	Acyl CoA Synthetase (ACSL 1)	Cytosolic	2180	601	1	-	1	1sl - 0.799	0	1	0
51	Carnitine Acyl Transferase	Cytosolic / MM	1384	601	1	1sl - 0.723	2	1sl - 0.816	1	2	3
52	Acyl CoA Dehydrogenase	MM	28976	803	13	11sl - 0.86 / 0.994 / 1.0 / 1.0 / 1.0 / 0.985 / 0.935 / 0.912 / 0.935 / 0.892 / 0.89	0	-	1	0	0
53	Enoyl CoA Hydratase	MM	1891	832	7	5sl - 0.844 / 0.902 / 0.922 / 0.893 / 0.744	1	1sl - 0.832	1	2	0
54	β Hydroxyacyl CoA Dehydrogenase	MM	3030	601	3	2sl - 0.98 / 0.968	3	1sl - 0.773	3	0	0
55	Thiolase 1	MM	38	679	6	5sl - 0.988 / 0.927 / 0.837 / 0.892 / 0.924	3	3sl - 0.808 / 0.775 / 0.706	2	2	0
56	Thiolase 2	MM	39	733	3	3sl - 0.985 / 0.9 / 0.843	1	-	2	1	0

NCBI Gene identification number (ID); Mitochondrial brown fat (MBF); *Cis*-element sites (sl); mitochondrial membrane (MM); number of recognized sites (Nº).

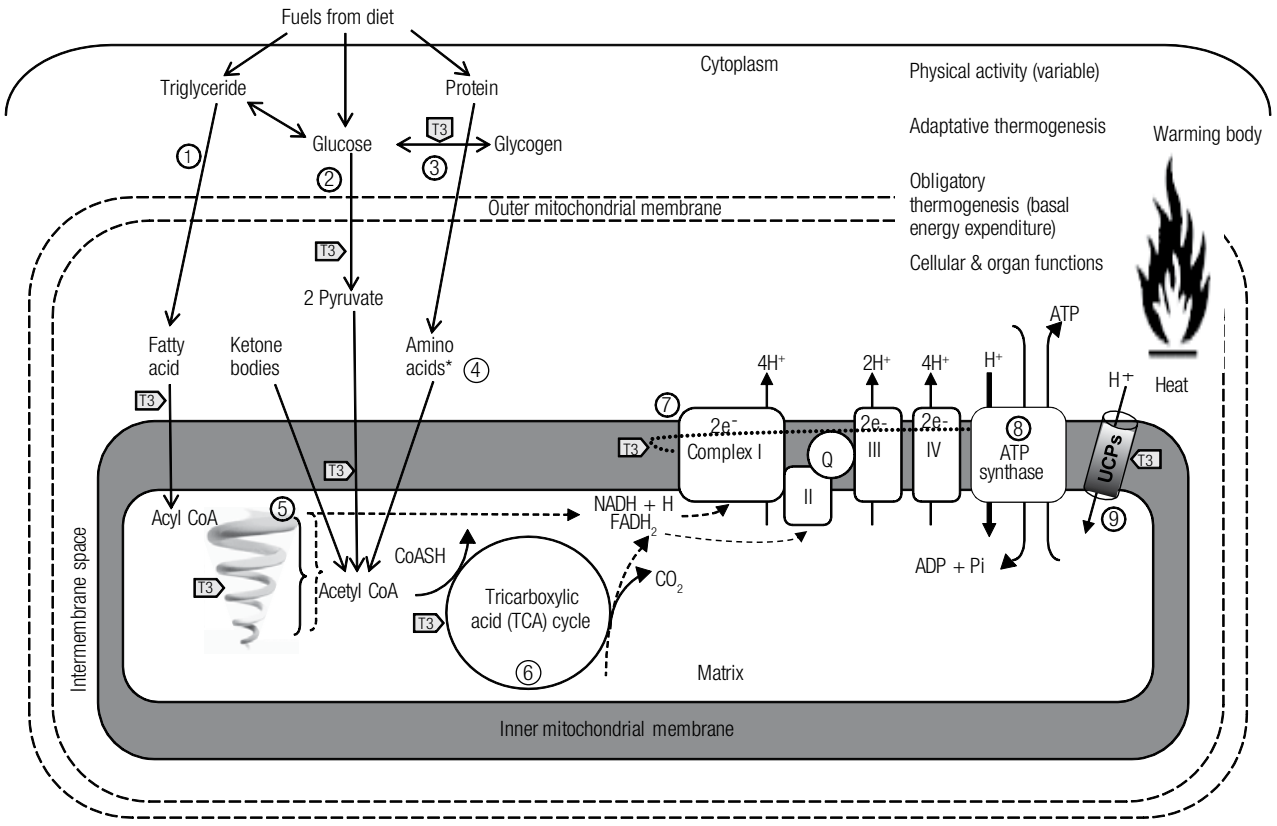

Figure 1. Schematic representation of the interplay between fuel oxidation routes and thermogenesis. **1,5**-lipid β-oxidation, **2-**glycolysis, **3-**glycogenolysis, **4-**proteolysis, **6**-Krebs cycle, **7-**respiratory chain, **8-**oxidative phosphorylation and **9-**uncoupling protein-meditated proton leak are potentially regulated by T3 on promoting heat generation. * Glycogenic amino acids.

Glucose phosphate isomerase (GPI, Gene ID: 2821) is located on 19q13.1 and belongs to the GPI family, which members encode multifunctional phosphoglucose isomerase proteins involved in energy pathways. We can expect that T3R/CREB regulation of GPI might increase glucose oxidation, thus generating more heat. In fact, GPI works as a dimeric enzyme that catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate, so in the cytoplasm, the gene product is involved in glycolysis and gluconeogenesis (27). Interestingly, GPI seems to work outside the cell as a neurotrophic factor for spinal and sensory neurons. Mild defects in this gene cause nonspherocytic hemolytic anemia, while its severe enzymatic deficiency can be associated with hydrops fetalis and immediate neonatal death associated with neurological impairment (28,29). GPI is differentially induced by a hypoxic environment as a transient adaptative response (30), as well as related to regulating cell proliferation, whereas its overexpression may increase *in vitro* neoplastic transformation (31).

Succinate dehydrogenase complex subunit C (SDHC, Gene ID: 6391) and succinate dehydrogenase complex subunit A (SDHA, Gene ID: 6389) have their genes located on 1q23.3 and 5p15, respectively. Each gene encodes one of four nuclear-encoded subunits that comprise succinate dehydrogenase, also known as the mitochondrial complex II, where other subunits of the complex anchor, thus forming the major catalytic core of succinate-ubiquinone oxidoreductase, placed in the inner mitochondrial membrane. In addition to participating in the aerobic respiratory chain of mitochondria, it is also a key enzyme complex of the tricarboxylic acid cycle (32). SDHC mutations have been associated with paragangliomas (33). Since alternatively spliced transcript variants of these genes have been described, we may also speculate if T3R/CREB would contribute to one of these transcriptional fates, especially if variations in basal metabolic rate and in climate are taken into account.

NADH dehydrogenase (ubiquinone) flavoprotein 1, also termed as *NDUFV1* gene (Gene ID: 4723) encodes the 51-kD subunit of complex I (NADH: ubiquinone

oxidoreductase) of the mitochondrial respiratory chain and it is located on 11q13. Microarray analysis reveals that *NDUFV1* is one of the six downregulated genes (among 297 genes) when obese and diabetic patients are fed with high fat diet (34). Interestingly each one of the six genes is known to be crucial for oxidative phosphorylation in the skeletal muscle; all of them work on regulation steps quite close to the terminal generation of ATP store. Thus, it is not surprising that T3R/CREB could transcriptionally modulate this metabolic route.

Cytochrome c oxidase (*COX4I1*, Gene ID: 1327) encodes the nuclear-encoded subunit IV isoform 1 – the terminal enzyme of the mitochondrial respiratory chain. It is part of a multi-subunit enzyme complex that couples the transfer of electrons from cytochrome c to molecular oxygen and contributes to a proton electrochemical gradient across the inner mitochondrial membrane. The whole complex consists of 13 mitochondrial- and nuclear-encoded subunits, being *COX4I1* located on 16q22-qter. In fact, this gene is genomically placed at the 3' of the *NOC4* (neighbor of *COX4I1*) gene in a head-to-head orientation, and curiously shares the promoter with it. Thus, it could be hypothesized that both genes are under T3R/CREB genome regulation as well as other mitochondrial enzymes pointed out in our *in silico* screening. Therefore, we suggest that this group of oxidation enzymes is potentially influenced by thyroid status, orchestrating the tone of metabolism either by the increase of thermodynamic chemical energy or by the increase of proton leak – a thermogenin (UCP) regulating role. Indeed, manipulating COX4I1 expression on cytochrome c oxidase activity, terminal ATP production, oxygen consumption, and reactive oxygen species generation indicates that this subunit switch is a homeostatic response which optimizes the efficiency of respiration at different oxygen concentrations, especially during temporary muscle hypoxia (35).

Another T3R/CREB possibly regulated enzyme is the mitochondrial uncoupling protein 5 (UCP5), which is a member of the larger family of mitochondrial anion carrier proteins (MACP) (36). The *UCP5* gene, also termed SLC25A14 – a brain mitochondrial solute carrier family 25, member 14 (Gene ID: 9016) is located in Xq24. As other UCPs, it deviates oxidative phosphorylation from ATP synthesis with energy released as heat, also referred to as the mitochondrial proton leak. Moreover, UCPs facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane, therefore altering pH gradient. The regulatory role of T3 on temperature acclimation has been postulated based on the observation of increased UCP3 expression in the skeletal muscle of rats kept over 8 weeks up to 8°C, and that UCP3 expression is decreased in rats acclimated to 30°C. Similar findings were verified in hyperthyroid rats compared with euthyroid controls (37).

Phosphoglucomutase 5 (PGM5, Gene ID: 5239) is a phosphotransferase enzyme involved in the interconversion of glucose-1-phosphate and glucose-6-phosphate, which has its gene located on 9q13 (38). Its activity is essential in the formation of carbohydrates from glucose-6-phosphate and in the formation of glucose-6-phosphate from galactose and glycogen. In fact, it plays a key role in the control of the metabolism towards glycolysis and gluconeogenesis, directing the biochemical fate according to the availability of energy stored as ATP (39,40).

In addition, the enzymes involved in lipid metabolism -acetyl-Coenzyme A acetyltransferase 1 (or acetoacetyl Coenzyme A thiolase 1, *ACAT1*, Gene ID: 38) and acetyl-Coenzyme A acetyltransferase 2 (or acetoacetyl Coenzyme A thiolase 2, *ACAT2* Gene ID: 39) were also pulled through the screening. They have their genes located on 11q22.3-q23.1 and 6q25.3, respectively. The first encodes a mitochondrial enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA, whereas the second gene encodes the cytosolic enzyme (thiolase 2). It has been suggested that ACAT reflects the metabolic status of the cell by the availability of acetyl molecules likely involved in insulin-mediated regulation (41). As described for cytochrome P450 expression (42), we could suggest that these genes are similarly involved in the T3 regulation of metabolism, even though further *in vitro* evidence is needed to support our initial insight on the T3R/CREB regulatory-participation hypothesis.

In accordance to our *in silico* findings, other *in vitro* evidence has indicated that T3-binding alters the conformation of the thyroid hormone receptor in such a way as to release the corepressor complex (inhibitory) and engage a coactivator complex that comprises multiple histone acetyltransferases, including a steroid receptor family coactivator, a p300/CREB-binding protein-associated factor (PCAF), and a CREB binding protein (CBP) (2). Taken all together, we were able to assemble this mechanism of partnership as illustrated in figure 2. We also warn that *cis*-elements for T3R and CREB on regulatory genomic sequences can still be located

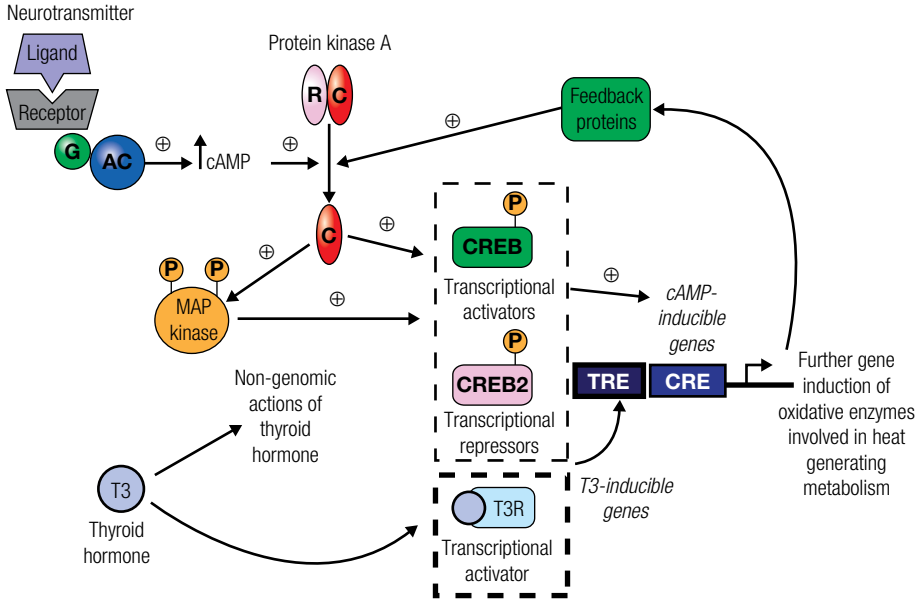

Figure 2. Assembly mechanism of T3R-CREB gene expression regulation of oxidative enzymes involved in heat generating metabolism.

a further 1.2Kb upstream of the potential promoter, even though the GENOMATIX program used in our study predicts the best sequence based on the conservation between orthologs, termed as golden promoter. Furthermore, all nucleotide sequences analyzed can be provided when requested to the authors.

In summary, our study identifies more enzymes in the complex spectrum of T3 genomic regulation over the existing ones. The abundance of functional T3-regulated promoters throughout all the metabolic pathways, either alone or with cAMP co-regulation, suggests that the contribution of the thyroid hormone to phenotypic thermogenesis variation and acclimation is likely to be appreciable. This *in silico* prediction is underscored by the additional impact of either the overt or subclinical, hyper- or hypothyroidism condition (43-45) on gene expression variation, related to thermogenesis. Therefore, this prediction sheds light on how thyroid hormone may influence the basal metabolic tune. It is particularly owing to such screening, and perhaps to certain associated roles (T3R/CREB) found in this work, that efforts are constantly being made, despite the well grounded hypothesis of the major T3-control of UCP expression, to validate *in vitro*, as yet unknown or unconfirmed joint roles in regulation, as the oxidative enzymes involved in heat generating metabolism.

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