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Letter

In Vivo Regulation of Human Skeletal Muscle Gene Expression by Thyroid Hormone

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Thyroid hormones are key regulators of metabolism that modulate transcription via nuclear receptors. Hyperthyroidism is associated with increased metabolic rate, protein breakdown, and weight loss. Although the molecular actions of thyroid hormones have been studied thoroughly, their pleiotropic effects are mediated by complex changes in expression of an unknown number of target genes. Here, we measured patterns of skeletal muscle gene expression in five healthy men treated for 14 days with 75 μg of triiodothyronine, using 24,000 cDNA element microarrays. To analyze the data, we used a new statistical method that identifies significant changes in expression and estimates the false discovery rate. The 381 up-regulated genes were involved in a wide range of cellular functions including transcriptional control, mRNA maturation, protein turnover, signal transduction, cellular trafficking, and energy metabolism. Only two genes were down-regulated. Most of the genes are novel targets of thyroid hormone. Cluster analysis of triiodothyronine-regulated gene expression among 19 different human tissues or cell lines revealed sets of coregulated genes that serve similar biologic functions. These results define molecular signatures that help to understand the physiology and pathophysiology of thyroid hormone action.

[The list of transcripts corresponding to up-regulated and down-regulated genes is available as a web supplement at http://www.genome.org.]

Thyroid hormones control essential functions in development and metabolism. The importance of the thyroid was recognized in the 19th century when an enlargement of the gland in hyperthyroidism was found to be associated with heart dysfunction, exophthalmos, and increased metabolic rate (Harrington 1935; Dauncey 1990). The isolation of thyroxine, and later of triiodothyronine (T3) a much more active molecule, resulted in a better understanding of the pleiotropic effects of the hormones and their therapeutic use. The major effects of thyroid hormones are mediated by modulation of gene transcription. Most of the characterized thyroid response elements in target genes are positive *cis*-acting elements at which gene transcription is repressed by unliganded thyroid hormone receptors (TRs) and activated by T3-occupied TRs (Wu and Koenig 2000; Zhang and Lazar 2000). In the presence of ligand, the TR undergoes a conformational change, which results in the replacement of a corepressor complex by a coactivator complex. The coactivator histone acetyltransferase activity leads to an open transcriptionally active chromatin state. The recruitment of the TR-associated protein complex may constitute a subsequent step in transcriptional activation by T3. In the absence of ligand, the heterodimer interacts with a corepressor complex with histone deacetylase activity. Histone deacetylation and DNA methylation both lead to transcriptional repression.

Although much has been learned about the molecular mechanisms of thyroid hormone action, a limited number of target genes has been identified. Most studies have focused on rodent liver (Feng et al. 2000). A large-scale profile of thyroid hormone transcriptional effects in vivo never has been undertaken in humans. In adults, thyroid hormones have a marked thermogenic effect and promote weight loss (Freake and Oppenheimer 1995; Rooyackers and Sreekumaran Nair 1997). Skeletal muscle is an important target of thyroid hormone action. It accounts for most of the variation in metabolic rate between individuals and plays a crucial role in protein metabolism (Zurlo et al. 1990). Here, we report the application of cDNA microarray technology to study the effect of thyroid hormone in vivo on human skeletal muscle. We defined a transcriptional profile of 383 genes regulated by T3. Most of these genes are novel targets of thyroid hormone. They belong to functional classes that explain the effect of T3 on protein turnover and energy metabolism. The data also

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reveal new mechanisms for the biologic action of T3, extending well beyond the classic metabolic effect of the hormone.

RESULTS AND DISCUSSION

In Vivo Treatment with Triiodothyronine and Use of cDNA Microarray on Skeletal Muscle mRNA

Nine healthy men received 75 µg/d of T3 for 14 days (Table 1). The treatment induced a 1.7-fold increase in free T3 levels. Free T4 levels and thyroid-stimulating hormone levels were decreased. The T3 treatment did not significantly modify body weight, fat mass, or lean body mass. Changes in gene expression induced by T3 therefore could be monitored independently of changes in body composition. Consistent with the well-known effect of thyroid hormones on energy expenditure, resting metabolic rate expressed in kilocalories per day or adjusted for lean body mass was increased by 13% and 15%, respectively. The respiratory quotient was decreased. As expected, heart rate and systolic (but not diastolic) blood pressure were increased by thyroid hormones.

Microbiopsies of vastus lateralis skeletal muscle were performed before and after the treatment. For microarray experiments, we used samples from five subjects. After amplification of total RNA (Wang et al. 2000a), fluorescently labeled cDNA was prepared from each experimental sample. For each subject, probes from basal and T3 treatment conditions labeled with cyanine (Cy) 3 or Cy5 dyes were hybridized to cDNA microarray. After a filtering procedure to eliminate bad-quality spots and background correction, $\log_2$ transformed data for each experiment were normalized and centered to the mean. Quantitative RT-PCR assays were performed on total RNA from eight subjects, of which four were not included in the microarray experiments.

Analysis of High-Density Microarray Data

We wanted to detect genes that show a statistically significant change in expression during thyroid hormone treatment. Most methods that have been used to analyze microarrays do not assess the degree to which significant changes in gene expression occurred by chance. Significant analysis of microarray (SAM) is a statistical method for accomplishing this task (Tusher et al. 2001). If there is no change due to treatment, then the true mean $\log_2$ ratio among the samples should be zero. Thus, we performed a t-statistic for each gene that tests whether the true mean is zero. SAM is a nonparametric method that decides how to call genes significant, and what the multiple testing error measure is for each significance region. The error measure is the expected proportion of false-positives among all genes called significant, called the false discovery rate (FDR). The SAM procedure was performed on 18,705 cDNAs for which signals were recovered in the five subjects. We found 21 cDNAs with estimated FDR of <0.1%, 98 with estimated FDR <10% and 449 with estimated FDR <15%.

Among the 449 cDNAs, 295 cDNAs were uniquely represented on the microarray. On the remaining 154 cDNAs, replicates were found within the 18,705 cDNAs. For all replicates corresponding to the same UniGene number, we tested, using Student's *t*-test, whether the expected value of the $\log_2$ ratio was 0. On the 133 genes with replicates, 88 genes showed consistent regulation by T3 for the various cDNAs ($P < 0.05$). It must be stressed that this selection procedure is stringent. Each of the mRNA variants of a gene produced by alternative promoters or splicing or polyadenylation sites has the same UniGene number but is not necessarily identically regulated by T3. The list of the 403 transcripts corresponding to 381 up-regulated and two down-regulated genes is presented as a Supplementary Information Table (available as an online supplement at http://www.genome.org). The up-regulated genes showed a mean fold change above 1.43. To test the validity of the array experiments and SAM procedure, we performed real-time quantitative RT-PCR on six genes. Five of the genes were selected randomly among the genes with greater than twofold up-regulation. The sixth gene encodes uncoupling protein 3 that was shown to be up-regulated by T3 in skeletal muscle (Gong et al. 1997; Barbe et al. 2001). For each gene, the data from quantitative RT-PCR confirmed the array data (Table 2).

Reconstruction of the human skeletal muscle transcriptional profile has revealed a set of ~400 genes highly expressed or specific of skeletal muscle (Pietu et al. 1999; Bortoluzzi et al. 2000). Two hundred forty-seven genes were represented on the array. We found that 42 (17%) genes were regulated by T3, showing the critical impact of thyroid hormone on genes characteristic of skeletal muscle. Because we assessed a large fraction of the transcriptome using high-density microarrays, the selected genes may reflect the main pathways regulated by T3 in human skeletal muscle. We assigned genes induced by T3 into functional categories (Table 3). We used Gene Ontology annotations available for 6331 cDNAs represented on the array to calculate the percentages of genes in each functional category and compared them with the percentages obtained for T3-regulated genes. Figure 1 shows that some categories are more represented in the T3-regulated gene group indicating that thyroid hormone has profound impact on these cellular pathways. Finally, to determine whether T3-regulated genes shared common patterns of expression, cluster analysis of the 403 transcripts was performed among 19 different human tissues and cell lines using a hierarchical clustering method (Eisen et al. 1998). Interestingly, a significant fraction of genes from functional categories highly regulated by T3,

Table 1. Clinical Data of Nine Healthy Men before and during Triiodothyronine Treatment

	Before	During (day 14)
Body weight (kg)	71.4 ± 3.9	70.7 ± 3.8
Fat mass (kg)	15.0 ± 1.7	15.4 ± 2.0
Lean body mass (kg)	53.2 ± 2.7	52.3 ± 2.5
Heart rate (beats/min)	58 ± 2	72 ± 4[a]
Systolic blood pressure (mm Hg)	116 ± 3	121 ± 4[b]
Diastolic blood pressure (mm Hg)	63 ± 2	67 ± 2
Resting metabolic rate (kcal/d)	1720 ± 55	1951 ± 93[b]
(J/min/kg lean body mass)	95 ± 3	109 ± 3[b]
Respiratory quotient	0.85 ± 0.02	0.78 ± 0.01[b]
Free T3 (pM)	6.8 ± 0.2	11.2 ± 0.9[a]
Free T4 (pM)	11.0 ± 0.9	4.1 ± 0.9[a]
TSH (mU/L)	2.1 ± 0.3	0.06 ± 0.01[a]
NEFA (µM)	301 ± 45	330 ± 32

NEFA, nonesterified fatty acid; TSH, thyroid-stimulating hormone. Data (means ± standard error) were compared using paired *t*-test.
[a]$P < 0.01$.
[b]$P < 0.05$.

Table 2. Fold Change of Human Skeletal Muscle mRNA Levels during Triiodothyronine Treatment

UniGene no.	Encoded protein	Microarray	Quantitative RT-PCR
Hs.21537	Protein phosphatase 1, catalytic subunit, β isoform	3.04	2.30 ± 0.45[a]
Hs.80595	NADH dehydrogenase (ubiquinone) Fe-S protein 5	2.54	1.54 ± 0.20[b]
Hs.131255	Ubiquinol-cytochrome c reductase binding protein	2.46	3.08 ± 0.89[b]
Hs.118836	Myoglobin	2.38	2.10 ± 0.52[b]
Hs.101337	Uncoupling protein 3	2.30	4.31 ± 1.44[b]
Hs.111779	Secreted protein acidic and rich in cysteine (SPARC)	2.10	1.99 ± 0.40[b]

Microarray experiments were performed on amplified RNA from five subjects. Quantitative RT-PCR were performed on total RNA from eight subjects. Quantitative RT-PCR data (means ± standard error) were compared using paired *t*-test.
[a]$P < 0.01$.
[b]$P < 0.05$.

such as energy metabolism, protein catabolism, protein synthesis, and ribonucleoprotein and RNA metabolism, is coexpressed in human tissues (Fig. 2). This suggests that thyroid hormone may participate in the transcriptional control of coregulated genes.

Global View at mRNA Expression Changes Induced by T3

A global view of the changes shows that thyroid hormone specifically affects many genes that are related to the physiological effects of the hormone on protein and energy metabolism (Table 3; Fig. 1). The largest fraction of regulated genes was involved in the transcriptional and post-transcriptional control of protein synthesis. One-fourth of the genes was gathered in a gene cluster (Fig. 2A), indicating common regulatory mechanisms of transcription for these genes. In vivo, multiple mechanisms therefore can account for the control of protein levels by T3. T3 increases the expression of numerous factors involved in transcriptional control, pre-mRNA processing, and protein translation (Table 3). Hence, protein synthesis may be promoted by thyroid hormone both directly through the direct effect of T3 on target gene promoters and indirectly through transcriptional control of proteins involved in transcriptional, post-transcriptional, and translational mechanisms (Fig. 3). Thyroid hormones are known to increase both skeletal muscle protein synthesis and degradation resulting in net protein breakdown (Rooyackers and Sreekumaran Nair 1997). Our data show a concomitant increase in mRNA expression of protein catabolism factors (Table 4). Most changes affected the ubiquitin/proteasome pathway, which is part of the non-lysosomal degradation of intracellular proteins. The protein turnover cluster (Fig. 2A) contained several subunits of the proteasome. Another important group

Table 3. Global Changes of Gene Expression Induced by Thyroid Hormone in Human Vastus Lateralis Skeletal Muscle

Functional classes	Number (%)
Transcriptional control	33 (8.7)
Transcription factors	
Coactivators/corepressors	
Histone (de)acetylation	
Protein synthesis	14 (3.7)
Ribosomal proteins	
Translation initiation factors	
Glucose and lipid metabolism	8 (2.1)
Glycogen synthesis and glucose utilization	
Signal transduction	36 (9.4)
Protein phosphatases	
Protein kinases	
G proteins	
G protein–coupled receptors	
Cytoskeleton	21 (5.5)
Ribonucleoproteins and RNA metabolism	16 (4.2)
Pre-mRNA processing	
Protein catabolism	20 (5.2)
Ubiquitin-proteasome pathway	
Mitochondrial energy metabolism	22 (5.8)
Respiratory chain proteins	
Mitochondrial carriers	
Citric acid cycle	
Cellular trafficking	13 (3.4)
Intracellular transport	
Cellular immunity	9 (2.4)
Miscellaneous	45 (12)
Expressed sequence tags	144 (38)

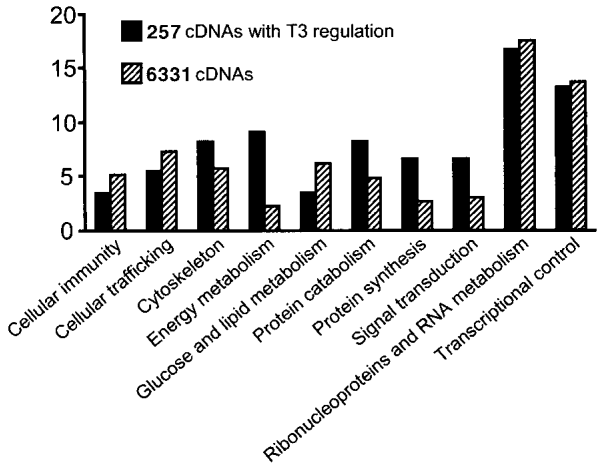

Figure 1 Impact of thyroid hormone on genes grouped in functional categories. Information on gene function was obtained from Gene Ontology annotations for 6331 cDNAs represented on the microarray. Among the genes regulated by thyroid hormone, information was available for 257 cDNAs. The cDNAs were classified into the functional categories defined in Table 1. Bars represent percentages in each category.

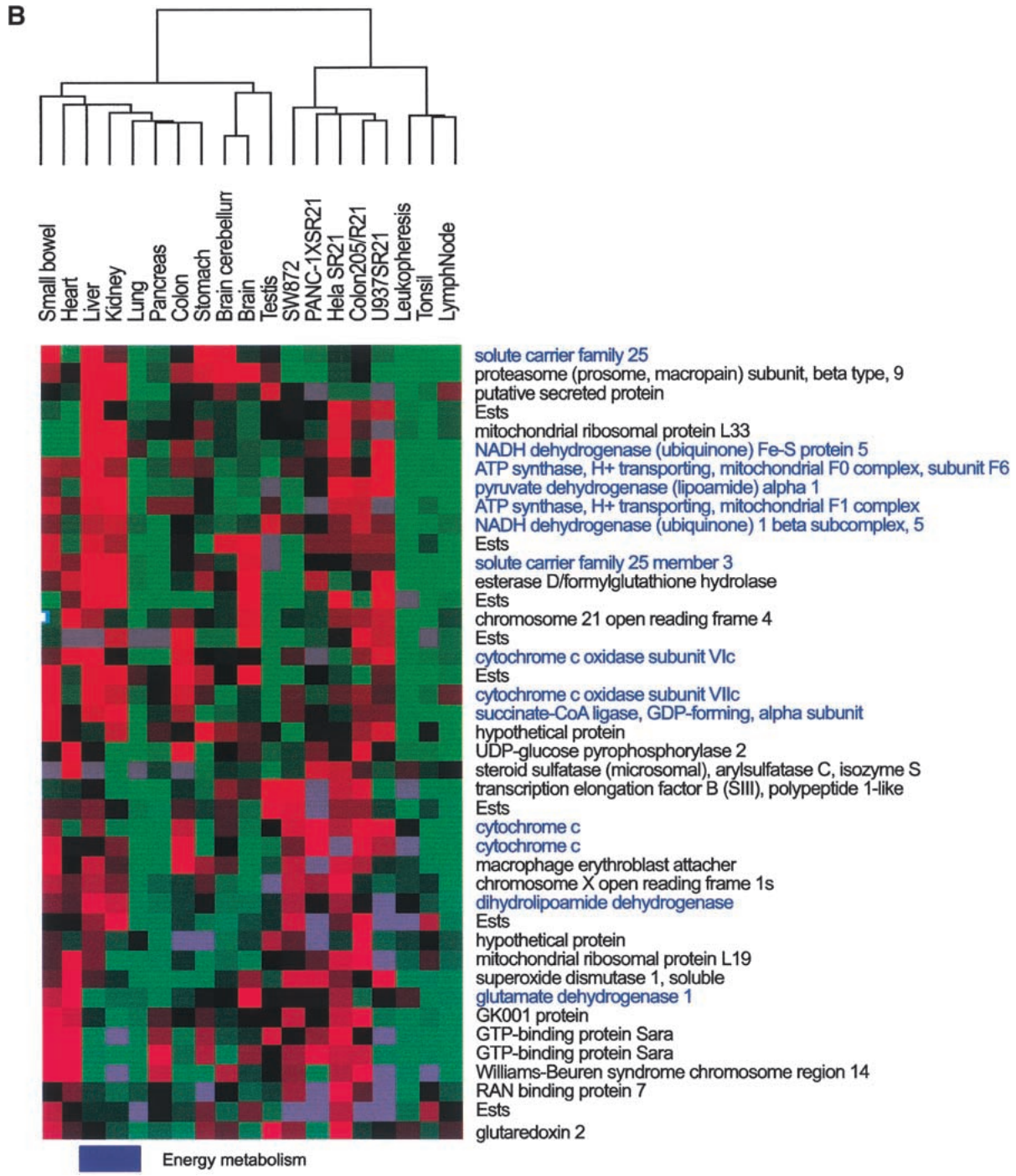

Figure 2 Two-dimensional clustering of 19 tissue experiments and 403 transcripts, which showed variation after triiodothyronine treatment. The set of genes were selected from the data matrix provided by the hybridization of 19 human tissues and cell lines to a common reference pool. Experiments and responsive genes were grouped by hierarchical clustering after centering the $\log_2$ ratios on the mean for all experiments. Each row represents a single gene and each column an experimental sample. For each sample, the ratio of the abundance of the transcripts of each gene to the mean abundance across all experiments is represented by the color of the corresponding cell in the matrix file. (green boxes) Transcript levels lower than the mean. (red boxes) Transcript levels higher than the mean. (black boxes) transcript level equal to the mean. (gray lines) missing data. Each node of the gene dendrogram was analyzed, and we focused on sets of genes clustered by functions. The upper dendrogram shows similarities in the expression pattern between tissues and cell lines. (*A*) Protein turnover cluster. (*B*) Energy metabolism cluster.

of genes up-regulated by T3 encoded proteins of energy metabolism (Table 5). Search in PubMed (National Center for Biotechnology Information) showed that nine of these genes were positively regulated by thyroid hormone in various mammalian tissues. A cluster gathered 13 of the 22 mitochondrial proteins involved in energy metabolism (Fig. 2B). These

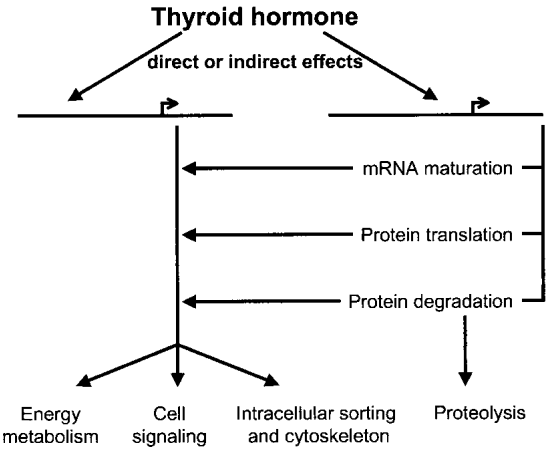

Figure 3 Summary of thyroid hormone regulation of gene expression in human skeletal muscle. The 14-day treatment with triiodothyronine induces direct and indirect effects on gene transcription. The hormone regulates genes with a wide range of cellular functions. Post-transcriptional regulation of protein expression also may contribute to the physiological and pathological action of thyroid hormone.

coregulations may contribute to the marked effect of T3 on skeletal muscle respiration (Tata et al. 1963).

Our study reveals that the transcriptional effect of T3 on skeletal muscle extends well beyond the classic metabolic effects of the hormone. Thirty-six signal transduction genes were up-regulated. Through induction of receptor, G protein, and protein kinase gene expression, thyroid hormone may exert its permissive effect on hormonal regulation of skeletal muscle metabolism. Moreover, T3 increased mRNA expression of catalytic and regulatory subunits of protein phosphatases 1 and 2A. T3 also may influence cellular trafficking and tissue remodeling through increased expression of genes involved in protein transport and maturation, cytoskeleton assembly, and exchange between intracellular organelles. Our data also identify 144 expressed sequence tags up-regulated by T3, which correspond to novel putative target genes.

Up-Regulation of Transcription Factors and Cofactors

Thyroid hormone up-regulated non-specific factors promoting transcription such as two subunits of transcription elongation factor B that assist RNA polymerase II as an auxiliary elongation factor. T3 induced mRNA expression of histone deacetylase 2 and DNA methyltransferase 3A, which are involved in transcriptional repression. This may constitute an indirect pathway for thyroid hormone–mediated mRNA down-regulation. Furthermore, T3 treatment resulted in up-regulation of nuclear factors involved in skeletal muscle transcriptional control. These effects may contribute to indirect control by T3 of numerous genes. MADS box transcription enhancer factor 2 and myogenic factor 6 are two transcription factors involved in the late skeletal muscle differentiation program (Braun et al. 1990; McDermott et al. 1993). LAZ3/Bcl6 is a transcriptional repressor important for myogenesis, possibly through the induction and stabilization of the withdrawal from the cell cycle (Albagli-Curiel et al. 1998). C/EBPβ is positively regulated by T3 in liver (Menendez-Hurtado et al. 1997). The transcription factor controls insulin signal transduction pathway in skeletal muscle (Wang et al. 2000b). The up-regulation of C/EBPβ in skeletal muscle therefore may contribute to the insulin-resistant state observed in thyrotoxicosis. The up-regulated genes also included nuclear receptor coactivator 4, a coactivator of androgen receptor and peroxisome proliferator-activated receptor (Yeh and Chang 1996).

Post-Transcriptional Control of Protein Turnover

Exposure to increased plasma level of thyroid hormone had profound effects on genes controlling post-transcriptional mechanisms including mRNA maturation and protein translation. Several transcripts encoding ribonucleoproteins and splicing factors were up-regulated. T3 treatment induced an increase in mRNA for protein synthesis factors such as ribosomal proteins and translation initiation factors including elF1A that is required for maximal rate of protein biosynthesis (Fletcher et al. 1999). Our data show an elevated level of mRNAs of the ubiquitin/proteasome pathway (Table 4). The ATP-dependent proteolytic pathway is responsible for the rapid degradation of many enzymes, signal transduction proteins, and transcriptional regulators including TR. Interestingly, proteasome-mediated degradation may play a critical role in the transcriptional activation of TR (Dace et al. 2000). We found that T3 induced an increase in mRNAs for proteasome subunits including C2, C6, and D12, which are part of the 19S complex of the 26S regulatory particle. There was also an increase in proteasome subunit A1, A3, A4, and A5 mRNAs that are essential α-type catalytic subunits of the 20S particle. In addition, T3 also increased mRNA for enzymes of the ubiquitinylation complex such as ubiquitin-conjugating enzyme 2B and cullin 2. Branched chain keto acid dehydrogenase E1, the rate-limiting enzyme in the catabolism of the branched chain amino acids, was up-regulated (Shimomura et al. 1995). Thus, hyperthyroidism appears to be accompanied by coordinated adaptations leading to an enhanced capacity of the proteasome degradative system. Combined with the increase in mRNA for ubiquitin-specific proteases, these regulations may be responsible for the loss of body protein mass under thyrotoxicosis (Ramsay 1965). Accordingly, increased proteolysis in skeletal muscle of hyperthyroid rats is mainly mediated via the ubiquitin-proteasome pathway (Tawa et al. 1997).

T3 Effect on Metabolism

Thyroid hormones participate with insulin and catecholamines in the regulation of skeletal muscle metabolism. The antagonism to insulin action was illustrated by the mRNA increase of the p85α phosphatidylinositol 3-kinase regulatory subunit. Indeed, mice lacking p85α show increased insulin sensitivity (Terauchi et al. 1999; Fruman et al. 2000). Thyroid hormones enhance the effect of catecholamines. An induction of the β_2-adrenergic receptor mRNA was observed consistent with the positive effects of T3 observed in human adipose tissue (Viguerie et al. 2002) and rat liver (Feng et al. 2000). As shown in hepatocytes (Swierczynski et al. 1991; Betley et al. 1993), T3 up-regulated enzymes involved in gluconeogenesis and glycogen metabolism (Table 5). Numerous genes of mitochondrial energy metabolism were up-regulated, including several enzymes associated with the citric acid cycle. Pyruvate dehydrogenase is one of the major enzymes responsible for the regulation of homeostasis of carbohydrate fuels in mammals. Two subunits of the complex, E1α and E3, were induced. An up-regulation of pyruvate dehydrogenase

Table 4. Thyroid Hormone–Induced Up-Regulation of mRNA Expression for Genes of Protein Catabolism

UniGene no.	Encoded protein	Fold
Hs.9280	Proteasome (prosome, macropain) subunit, β type, 9	3.20
Hs.61153	Proteasome (prosome, macropain) 26S subunit, ATPase, 2	2.56
Hs.79137	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase	2.15
Hs.811	Ubiquitin-conjugating enzyme E2B (RAD6 homolog)	2.14
Hs.77578	Ubiquitin-specific protease 9, X chromosome	2.10
Hs.79357	Proteasome (prosome, macropain) 26S subunit, ATPase, 6	2.06
Hs.82159	Proteasome (prosome, macropain) subunit, α type, 1	2.04
Hs.4295	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12	2.02
Hs.12272	Beclin-1	1.95
Hs.76913	Proteasome (prosome, macropain) subunit, α type, 5	1.93
Hs.251531	Proteasome (prosome, macropain) subunit, α type, 4	1.88
Hs.86978	Prolyl endopeptidase	1.87
Hs.78950	Branched chain keto acid dehydrogenase E1, α polypeptide	1.85
Hs.167108	Proteasome (prosome, macropain) subunit, α type, 3	1.83
Hs.99819	Ubiquitin-specific protease 16	1.81
Hs.82919	Cullin 2	1.79
Hs.75981	Ubiquitin-specific protease 14 (tRNA-guanine transglycosylase)	1.74
Hs.79357	Proteasome (prosome, macropain) 26S subunit, ATPase, 6	1.68
Hs.15303	Protein similar to mouse ubiquitin-protein ligase E3-α	1.66
Hs.101408	Branched chain aminotransferase 2, mitochondrial	1.55

The fold increase represents the mean of the five comparisons.

kinase 4, which is highly expressed in skeletal muscle, also was observed as shown in rat heart (Sugden et al. 2000). Phosphorylation of pyruvate dehydrogenase by the kinase results in inactivation and may be considered as an adaptive mechanism to enhance the use of fatty acids as an energy source. For the biogenesis of the respiratory apparatus, more than 100 proteins are necessary (Pillar and Seitz 1997). Most of them are encoded in the nucleus with only 13 being encoded by the mitochondrial genome. Among the 50 independent mRNAs representing respiratory chain proteins on the microarray, 13 were up-regulated during T3 treatment (Table 5) and seven of them were coexpressed in human tissues (Fig. 2B). The up-regulated genes included several subunits of NADH : ubiquinone oxidoreductase (complex I) where proton translocation is coupled to electron transfer. Cytochrome c, a component of complex IV that donates electrons to the cytochrome oxidase complex, was markedly up-regulated as shown in rat skeletal muscle (Stevens et al. 1995). We observed an increase of two cytochrome oxidase (complex IV) and four ATP synthase (F1F0 ATPase or complex V) subunit mRNAs. The proteins of the respiratory chain need to be available in stoichiometric amounts for proper assembly in the inner mitochondrial membrane. T3 is a potent inducer of a subset of, but not all, nucleus-encoded respiratory chain genes (Wiesner et al. 1992). This suggests that thyroid hormone yields an increase of the other components, indirectly through post-transcriptional mechanisms. The increase in genes encoding protein translation factors (see above) may participate in this mechanism. Moreover, T3 induced genes involved in mitochondrial protein translation such as the only known mitochondrial translation initiation factor MTIF2 (Ma and Spremulli 1996) and mitochondrial ribosomal proteins S4, L3, and L19. Besides coupled respiration, thyroid hormones also increase uncoupled respiration and the leak of protons across the inner mitochondrial membrane (Lanni et al. 1999). In humans, T3 treatment of young adults for 3 days promotes in vivo mitochondrial energy uncoupling in skeletal muscle (Lebon et al. 2001). Here, we show that T3 induced three putative candidates to explain the uncoupling effect : uncoupling protein 3 and adenine nucleotide translocases 1 and 2 (Skulachev 1999). Genes of energy metabolism recently have been shown to be down-regulated during caloric restriction in vastus lateralis muscle of male rhesus monkeys (Kayo et al. 2001). Caloric restriction is characterized by a decrease in plasma thyroid hormone level that leads to a decrease of the metabolic rate. Our data therefore are consistent with a role of thyroid hormone in caloric restriction-induced changes in energy metabolism gene expression.

Modulation of Cytoskeletal Protein Expression

Thyroid hormone modulated expression of genes involved in the maintenance of cellular architecture. In human skeletal muscle, several adhesion complexes are essential for the organization of the actin cytoskeleton and maintenance of intercellular junctions (Chothia and Jones 1997). The mRNA for integrin α5 was up-regulated under T3. Integrins are receptors for extracellular matrix-mediated cytoskeletal organization and cell adhesion. Up-regulation also was observed for α and β-catenin mRNA. β-Catenin links the cadherin receptors to the actin cytoskeleton via α-catenin. In striated muscle, the dystrophin–glycoprotein complex forms a critical link between the cytoskeleton and the extracellular matrix (Matsumura et al. 1999). The mRNAs of the glycoproteins dystroglycan 1 and β-sarcoglycan were up-regulated. Ankyrins are protein linkers between the integral membrane proteins and spectrin-based cytoskeleton (Rubtsov and Lopina 2000). Through their interaction with cytoskeleton proteins such as vimentin or tubulin, they participate in the attachment of the intermediary filaments and microtubules to the membrane. In myocytes, ankyrin G is the main ankyrin form. Both ankyrin G and spectrin were positively regulated under T3. The expression of other molecules of the cytoskeleton was induced such as the actin-related protein ARPC2, tropomodulin, tubulin, and pinin, a desmosome-associated protein. Taken together, the up-regulation

Table 5. Thyroid Hormone–Induced Up-Regulation of mRNA Expression for Genes Involved in Metabolism

UniGene no.	Encoded protein	Pathway	Fold
Hs.77837	UDP-glucose pyrophosphorylase 2	Glycogen synthesis	3.02
Hs.1691	Glycogen branching enzyme	Glycogen synthesis	2.82
Hs.169428	Cytochrome c[a]	Respiratory chain (complex IV)	2.68
Hs.2043	Adenine nucleotide translocase 1	Mitochondrial carrier	2.66
Hs.33084	Fructose transporter GLUT 5[a]	Glucose metabolism	2.66
Hs.78713	Adenine nucleotide translocase 2[a]	Mitochondrial carrier	2.58
Hs.74635	Dihydrolipoamide dehydrogenase (pyruvate dehydrogenase complex E3)	Citric acid cycle	2.58
Hs.80595	NADH dehydrogenase (ubiquinone) Fe-S protein 5	Respiratory chain (complex I)	2.54
Hs.8364	Pyruvate dehydrogenase kinase, isoenzyme 4[a]	Citric acid cycle	2.52
Hs.19236	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 5	Respiratory chain (complex I)	2.50
Hs.131255	Ubiquinol–cytochrome c reductase binding protein	Respiratory chain (complex III)	2.46
Hs.155433	ATP synthase, F1 complex, γ 1	Respiratory chain (complex V)	2.35
Hs.101337	Uncoupling protein 3[a]	Mitochondrial carrier	2.30
Hs.81634	ATP synthase, F0 complex, subunit b, isoform 1	Respiratory chain (complex V)	2.26
Hs.109646	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 6	Respiratory chain (complex I)	2.23
Hs.61255	Fructose-1,6-bisphosphatase 2[a]	Neoglucogenesis	2.13
Hs.1023	Pyruvate decarboxylase (pyruvate dehydrogenase complex E1-α)	Citric acid cycle	2.10
Hs.429	ATP synthase, F0 complex, subunit c, isoform 3	Respiratory chain (complex V)	2.09
Hs.79876	Steroid sulfatase, arylsulfatase C, isozyme S[a]	Steroid hormone synthesis	2.02
Hs.73851	ATP synthase, F0 complex, subunit F6	Respiratory chain (complex V)	2.02
Hs.78060	Phosphorylase kinase, β	Glycogenolysis	2.00
Hs.268012	Long-chain fatty acid–coenzyme A ligase 3	Lipid β oxidation	1.99
Hs.7043	Succinate-CoA ligase, GDP-forming, α subunit	Citric acid cycle	1.99
Hs.74823	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 1	Respiratory chain (complex I)	1.96
Hs.3462	Cytochrome c oxidase subunit VIIc	Respiratory chain (complex IV)	1.90
Hs.76688	Carboxylesterase 1	Cholesterol metabolism	1.86
Hs.183435	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 1	Respiratory chain (complex I)	1.82
Hs.74649	Cytochrome c oxidase subunit VIc[a]	Respiratory chain (complex IV)	1.74
Hs.77508	Glutamate dehydrogenase 1[a]	Citric acid cycle	1.72
Hs.177584	3-oxoacid CoA transferase	Citric acid cycle	1.64

The fold increase represents the mean of the five comparisons.
[a]Proteins that have been shown to be positively regulated by thyroid hormones in rodents.

of genes encoding proteins of various adhesion complexes may contribute to skeletal muscle remodeling under thyrotoxicosis.

Conclusion

In this article, we have characterized a transcriptional profile in response to T3 in vivo. Sorting of up-regulated genes into functional classes and determination of common expression patterns in human tissues defined the molecular signatures that underlie the pleiotropic effect of thyroid hormone in human skeletal muscle (Fig. 3). In line with the known physiological effects of T3, induction of many genes involved in protein turnover and energy metabolism was observed. The study also reveals novel target cellular pathways. The impact on these pathways may help to understand the permissive effect of T3 on signal transduction cascades, intracellular transport, and tissue remodeling. This study also illustrates the value of a coupled use of DNA microarrays with microbiopsies from human tissues to study in vivo the physiological and pathological action of hormones.

METHODS

Subjects and Clinical Protocol

Nine healthy male Caucasian volunteers (22–33 years old) were recruited. The study was performed according to the Huriet Law and INSERM Good Clinical Practice guidelines. The protocol was approved by the Ethics Committee of Toulouse University Hospitals. Written informed consent was obtained from the subjects. All the visits and investigations were performed at the Toulouse Clinical Investigation Center. The same investigations were performed at day 0 and day 14. Participants were instructed to take one tablet of 25 μg of T3 (Cynomel; Marion Merell) three times a day (75 μg per day) during 14 days. No concurrent medication was allowed during the course of the study. The subjects were instructed by a dietitian to continue with their usual diet. After an overnight fast, a catheter was inserted at 8 a.m. into the antecubital vein for blood sampling and kept patent with isotonic saline. Three 10-min interval blood samples were drawn for determinations of metabolic and hormonal parameters. Heart rate and blood pressure were measured at three 10-min intervals using an automated blood pressure monitor. After a 1-h resting period in supine position, oxygen consumption (VO_2) and carbone dioxide production (VCO_2) were monitored over 30 min using an open-circuit ventilated-canopy system (Deltatrac II monitor; Datex Instrumentarium Corp.) calibrated with a reference gas. Resting metabolic rate was derived from VO_2 and VCO_2 using indirect calorimetry. Then, a percutaneous biopsy of the vastus lateralis muscle was performed using Weil Blakesley pliers. Approximately 3 mL 1% lidocaine (Xylocaine; Astra France) was injected into the skin and superficial tissue before the biopsy. The procedure involved a 5-mm incision through the skin and muscle sheath 15–20 cm above the knee. Muscle samples were flash-frozen in liquid nitrogen and stored at −80°C until analysis. On day 14, biopsies were performed on the contralateral

side. Before and at the end of the treatment period, body composition was assessed by dual-energy X-ray absorptiometry performed with a total body scanner (DPX, software 3.6; Lunar Radiation Corp.).

mRNA Quantitation

Total RNA was extracted using the RNA STAT-60 isolation reagent (Tel-Test). Real-time, quantitative RT-PCR was performed on GeneAmp 5700 Sequence Detection System (Applied Biosystems). A set of primers was designed for each gene using the software Primer Express 1.5 (Applied Biosystems). Amplicons of 65–90 base pairs with Tm between 79 and 82°C were selected. Reverse transcription was performed with 1 µg of total RNA for each biopsy, and 10 ng of cDNA was used as template for real-time PCR as recommended by the manufacturer. A dissociation curve was generated at the end of the PCR cycles to verify that a single gene product was amplified. A standard curve for each primer pair was obtained using serial dilutions of human skeletal muscle cDNA. We used 18S ribosomal RNA as control to normalize gene expression using the Ribosomal RNA Control TaqMan Assay kit (Applied Biosystems).

RNA Amplification and Microarray Experiments

Because the amount of total RNA obtained from skeletal muscle microbiopsies is limited, we used a two round amplification protocol to produce aRNA from total RNA (Wang et al. 2000a). The method, which is not based on PCR, ensures high-fidelity mRNA amplification. We prepared the aRNAs from 1.5 µg of total RNA in DNase-free water containing 1 µg of oligo-dT(15)-T7 primer. After denaturation, T7 bacteria phage promoter was incorporated into cDNA in a reverse transcription reaction containing a template-switch primer. cDNA synthesis was obtained after 90 min at 42°C. Full-length cDNA was synthesized by adding DNase-free water, Advantage PCR buffer and cDNA polymerase (Clontech), dNTPs, and RNase H (Promega). Reactions were terminated by incubation in a 1 M NaOH solution with 2 mM EDTA at 65°C. After cDNA extraction by phenol-chloroform-isoamyl alcohol and ethanol precipitation, cDNAs were resuspended in 60 µL water, passed through a Bio-6 chromatography column (Bio-Rad) and washed. In vitro transcription at 37°C for 6 h was performed using the T7 Megascript Kit (Ambion). RNA recovery was achieved by TRIzol purification (Life Technologies). Then, aliquots of aRNA (1 µg) were reverse-transcribed into cDNA using 2 µg of random hexamers. The reaction mixture was heated to 65°C for 10 min, and the synthesis was continued at 42°C for 90 min with Superscript II (Life Technologies). A second-strand cDNA synthesis and the in vitro transcription of aRNA were conducted as for the first round. Detail protocols of the hybridization and scanning procedure are described at http://cmgm.stanford.edu/pbrown/protocols/index.html. Briefly, 6 µg of aRNA was labeled by incorporating a Cy dye during the random primed reverse transcription with Superscript II. Amplified RNA from individuals before T3 administration were labeled with Cy3 and aRNA from individuals after T3 were labeled with Cy5, except for one subject with an inversed labeling. The labeled cDNA mixture was concentrated using microcon 30 column (Millipore) after the addition of human cot-1 DNA (Life Technologies). After denaturation, the probe was added to the array, which was covered by a Coverslip. The slide then was placed in a sealed humidified hybridization chamber for a 16-h hybridization at 65°C. Slides were washed twice in 2 × SSC 0.1% SDS, 1 × SSC, and then 0.5 × SSC. The arrays were immediately scanned using a GenePix 4000A confocal Scanner (Axon Instruments). Images were analyzed using GenePix pro 3 software. Data files generated by Genepix were entered into the Stanford Microarray Database (http://genome-www4.stanford.edu/MicroArray/SMD/). We applied a uniform scale factor to all measured intensities that normalized signal intensities between the two fluorescent images. This normalization factor was chosen so that the mean $\log_2$ (Cy5/Cy3) for a subset of good-quality spots (~16,000 spots) was 0.

Data Analysis

Before extraction of $\log_2$ ratio data, we applied a filtering procedure by omitting manually flagged elements (i.e., bad-quality spots). After also eliminating the spots with an average intensity below 1.5-fold above the background, 22,640 spots were recovered. The overall background was low because 85.5% of the spots had channel intensities fourfold above the background. To assess reproducibility of two hybridizations after independent amplifications, we compared aRNA preparations labeled with Cy3 and Cy5 from two different individuals before the treatment. The correlation coefficient between Cy3-labeled aRNA and Cy5-labeled aRNA was 0.93. We then extracted the $\log_2$ Cy5/Cy3 ratios (treated/untreated) for the five experiments. cDNA with missing data were excluded. Data were analyzed using the SAM procedure, a validated statistical technique for identifying differentially expressed genes across high-density microarrays (Tusher et al. 2001). Before calculations, the data from each of the five experiments were normalized in log-space to have mean 0 and standard deviation 1. In the SAM procedure, the modified t-statistic $d(i) = x(i)/[s(i) + S_0]$ is calculated for the i^{th} gene, where x(i) is the mean of the $\log_2$ ratio data across all five experiments, and s(i) is the appropriately scaled standard deviation. The quantity S_0 is an adjustment factor derived from the data, which attempts to make d(i) independent of s(i). Because the null hypothesis was that there was no treatment effect, we tested whether the expected value of the $\log_2$ ratio is 0 in the statistic d(i). SAM is a nonparametric procedure that compares the ordered d(i) to the expected value of the ordered statistics calculated under random assignment of treatment in the $\log_2$ ratio and calls genes significant based on this comparison. Testing all genes simultaneously requires one to implement a multiple comparison procedure, which guards against many false-positives. The FDR method controls the expected value of the ratio of the number of false-positives to the total number of genes called significant (Benjamini and Hochberg 1995). SAM provides a point estimate of the FDR based on the number of significant genes in randomized data and the original data. Details on the SAM procedure is available at http://www-stat.stanford.edu/~tibs/SAM/index.html. With an estimated FDR of 15%, 449 cDNA were selected as being differentially expressed. The channel intensities for this set of genes were more than twofold above the background, and 74% of them had channel intensities at least sixfold above the background. For 154 cDNAs with replicates among the 18,705 cDNAs, that is, the same UniGene number, we tested, using Student's *t*-test, whether the expected value of the $\log_2$ ratio was 0.

Coexpression of T3-Regulated Genes in Various Tissues and Cell Lines

We analyzed how the 403 transcripts might be coexpressed together in 19 human adult tissues and cell lines. Each tissue or cell line polyA$^+$ RNA-labeled with Cy5 was hybridized against a common reference pool consisting of 11 different cell lines described elsewhere (Perou et al. 2000). We extracted the Cy5/Cy3 ratios for the 403 transcripts and applied two-dimensional hierarchical clustering to the expression data (Eisen et al. 1998). Data were centered to the mean by subtracting the arithmetic mean of all ratios measured for each gene. We performed a hierarchical cluster analysis on both genes and experiments using the Pearson correlation coefficient as a

measure of similarities and average linkage clustering. The results were visualized by the Tree view software (http://rana.lbl.gov/).

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