Hepatic gene expression patterns in thyroid hormone-treated hypothyroid rats

J M Weitzel, S Hamann1, M Jauk, M Lacey1, A Filbry, C Radtke, K A H Iwen, S Kutz, A Harneit, P M Lizardi1 and H J Seitz

Institute of Medical Biochemistry and Molecular Biology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany
1Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520, USA

(Requests for offprints should be addressed to J M Weitzel; Email: weitzel@uke.uni-hamburg.de)

(J M Weitzel and S Hamann contributed equally to this work)

Abstract

Thyroid hormone (T3) is essential for normal development, differentiation and metabolic balance. We have performed DNA microarray experiments using hepatic RNA from hypothyroid and T3-treated hypothyroid rats in order to characterize T3-induced gene expression patterns after various time points (6, 24 and 48 h after the administration of the hormone). Sixty-two of 4608 different genes displayed a reproducible T3-response, and cluster analysis divided these differentially regulated genes into six expression patterns. Thirty-six genes were not significantly regulated within the first 24 h. Transient transfection experiments of eight late-induced gene promoters failed to detect a thyroid hormone response element within their regulatory elements, suggesting an indirect activation mechanism(s). In search for an intermediate factor of T3 action, we examined whether various rather ubiquitous transcription factors, peroxisome proliferator-activated receptors (PPARs) and coactivators of the PPARγ coactivator 1 family (PGC-1) are regulated by T3. Only PPARγ and PERC/PGC-1β exhibit a significant T3-response within the first 6 h after treatment, identifying these factors as candidate components for mediating the late-induced expression pattern. Regulation of early-induced genes within the first 6 h after administration of T3 on transcript levels correlates with altered protein levels after 24 and 48 h in vivo.

Journal of Molecular Endocrinology (2003) 31, 291–303

Introduction

Thyroid hormone (T3) has a profound influence on normal development, differentiation, and metabolism (Yen 2001). After a single injection of T3 to hypothyroid rats the metabolic rate remains unaffected for the first 20–30 h. After that period, the metabolic rate increases and reaches maximum levels between 72 and 96 h (Tata et al. 1963). This delayed response is surprising since nongenomic effects of T3 are already detectable within minutes (Davis et al. 2002) and genomic effects of T3 are typically detectable within a few hours (Weitzel et al. 2003). For many of the latter genes functional thyroid hormone response elements (TREs) have been reported (Zilz et al. 1990,Weitzel et al. 2001a), indicating a direct mechanism via binding of thyroid hormone receptors (TRs), which are ligand-regulated transcription factors. TRs modulate gene activity through alternately silencing or activating transcription by recruitment of either corepressor or coactivator complexes depending on the absence or presence of thyroid hormone (Robyr et al. 2000, Zhang & Lazar 2000).

However, several T3-target genes are expressed ‘late’ (24–48 h) after the administration of T3 in vivo, including cytochrome c (Scarpulla et al. 1986) and adenine nucleotide translocator 2 (ANT2) (Dümmler et al. 1996) and, noteworthy, an interaction of TR (via a TRE) with these target genes has not been observed (Li et al. 1997, Weitzel et al. 2003). These observations suggest an induction mechanism via the activation of an intermediate factor(s). Candidate proteins have
been identified which probably serve as intermediate factors, e.g. the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2, Scarpulla 2002) or the nuclear receptor coactivator PGC-1α, which has been shown to interact with NRF-1 (Wu et al. 1999, Knutti & Kralli 2001, Puigserver & Spiegelman 2003). Indeed, binding sites for NRF-1 and/or NRF-2 were detected in several target genes which are known to be regulated by thyroid hormone more than 12 h after the administration of T3 in vivo (Scarpulla et al. 1986, Luciakova & Nelson 1992, Scarpulla 2002, Weitzel et al. 2003). This finding strengthens the argument for an indirect T3-activating mechanism via an intermediate factor(s) since these factor(s) need to be synthesized before they can act.

Recently, we detected novel T3-target genes and identified two T3-mediated gene expression patterns after the administration of T3 to hypothyroid rats using cDNA expression arrays. DNA microarrays are powerful tools to analyse differential gene expression and were successfully used to identify novel T3-target genes in mouse, rat, human, cell line and tumour (Feng et al. 2000, Miller et al. 2001, Clement et al. 2002, Flores-Morales et al. 2002, Weitzel et al. 2001b, Wood et al. 2002). However, a large-scale analysis of T3-mediated temporal gene expression patterns has not been performed. Our previous study was hampered by the limited size of the arrays which contained 588 different genes. To receive a more representative set of T3-mediated gene expression patterns we now extended this study to a larger DNA microarray comprising 4608 different probes. In search of an intermediate factor for mediating the late T3-mediated gene expression pattern we extended our study to: (i) transcription factors which are known to be important for the activity of several late-induced T3-target genes, (ii) to peroxisome proliferator-activated receptors (PPARs), since many newly identified T3-target genes contain a functionally characterized PPAR response element, and (iii) to the coactivators PRC and PERC/PGC-1β.

Materials and methods

Animals and treatments

Adult male Wistar rats (Charles River Wiga, Charles River Deutschland GmbH, Sulzfeld, Germany) were housed under controlled conditions (22 °C, constant humidity, 12 h:12 h dark–light cycle); food and water were provided ad libitum. Rats were maintained in the animal care facility of the University Hospital Hamburg-Eppendorf according to the UKE animal care guidelines. Hypothyroidism was induced by i.p. injection of Na131I (Amersham Pharmacia) (250 µCi/100 g body weight) 28 days before the experiments. Hyperthyroidism was provoked by an intraperitoneal injection of T3 (50 µg/100 g body weight; Henning) which was repeated after 24 h. Thyroid hormone status was verified by measurements of serum T3, T4 and TSH levels: (hypo: T3 <0.4 µg/l; hyper: T3 >6.5 µg/l). Zero, 6, 24 and 48 h after thyroid hormone injection, rats were killed by decapitation and the livers were isolated for RNA and protein preparation.

RNA isolation and cDNA labelling

Total RNA was prepared from rat livers using RNA-Clean (Hybaid AGS, Heidelberg, Germany) following the manufacturer’s instruction and treated with DNase I (Clontech) (50 U, 30 min, 37 °C). One hundred microgram of total RNA (combined from identical amounts of RNA from three different animals per time point to minimize the effect of individual variation of the expression pattern) were labelled by direct incorporation of either Cy3-dUTP or Cy5-dUTP in a reverse transcriptase reaction (Eisen & Brown 1999).

Microarray experiments

Probes were hybridized to Mouse 4·6k poly-L-lysine cDNA microarrays (Yale Keck Microarray Facility) according to the Childs/Kucherlapati protocol (http://sequence.aecom.yu.edu/bioinf/microarray/protocol4.html). DNA microarrays containing 4608 cDNA fragments (obtained from the IMAGE consortium) were utilized which were not enriched in ‘classical’ T3-target genes and were printed in duplicates. We made an effort to set up a high standard, both experimentally and in the data analysis, in order to maximize the data quality. For example, we tried to avoid the effect of putative artificial signals caused by non-specific dye–cDNA interactions by conducting each microarray hybridization twice, where the second experiment was performed with a reversed Cye-dye.
assignment (the so called ‘dye flip’). Moreover, we combined the 6 h with the 24 h and the 24 h with the 48 h samples in a way that made it possible to cross-reference the data. Only signals that showed at least a ratio of 2 or 0.5 in all four independent microarray experiments (resulting in a total of eight data points) were considered up- or down-regulated respectively. Because of the reported problems with IMAGE consortium mouse cDNA clones (Halgren et al. 2001), gene identities were first confirmed by sequence analysis. After overnight incubation slides were scanned on a ScanArray 5000 microarray scanner (GSI Lumonics, Farmington Hills, MI, USA) and the generated 16-TIFF images were analyzed by QuantArray microarray analysis software (GSI Lumonics). Cluster analysis and visualization was performed using the Eisen Cluster and Treeview software respectively (Eisen et al. 1998).

**Northern blotting**

For northern blots 10 µg total RNA, combined from identical amounts of three animals per time point, were analyzed and normalized to: (i) the ethidium bromide staining signal and (ii) to the ubiquitin hybridization signal as described previously (Weitzel et al. 2001b). Fold increases were determined from T3-treated hypothyroid rat signal values (6, 24 and 48 h) divided by hypothyroid rat signal values within the same experiment. For hybridization, cDNA probes of particular genes (200–300 bp in length) were generated by RT-PCR amplification, using primers designed to the sequences described in Table 1. The portion of the cDNA which was used as probe for northern hybridization was (whenever possible) not identical to the portion of the cDNA that was used for the production of the microarray. Thus, we generated hybridization probes that served as a sequence-independent verification of the microarray hybridization data. The amplification products were gel-purified, ligated into pGEM T-easy (Promega) and verified by sequencing.

**Quantitative PCR**

Quantitative real-time RT-PCR was performed as described previously (Weitzel et al. 2001b). Again, PCR primers were (whenever possible) selected from a portion of the cDNA which was not used for printing of the arrays. PCR reactions were carried out in duplicates or triplicates with the following PCR primers: CREB for: 5’-ctatcatcagctgtacagtc-3’; CREB rev: 5’-tgtctcaagcttctgctgc-3’; HCF for: 5’-acagatgttagtggtctgc-3’; HCF rev: 5’-agttaggg cgtacagtgtgc-3’; HNF-4α for: 5’-tcatctcatgtggacctt cc-3’; HNF-4α rev: 5’-acacacatgctgtaggtgc-3’; mtTFA for: 5’-tgaaatgggctgttggtgc-3’; mtTFA rev: 5’-ctagctcatgcatctgagagac-3’; Sp1 for: 5’-cattggt gc-3’; YY1 for: 5’-gtagaagctgagctgc-3’; YY1 rev: 5’-ttttggtcttgatgcttcgc-3’; PPARα for: 5’-ctacag acatggagacctg-3’; PPARα rev: 5’-catagggacagctaa cttgg-3’; PPARβ for: 5’-ccagctgtcaggtgctgc-3’; PPARβ rev: 5’-tcatctcatgctgttgctgc-3’; PPRγ for: 5’-tattctcaggtgagctgc-3’; PPRγ rev: 5’-ccagctgtcaggtgctgc-3’; HCF for: 5’-ctctcaggtgagctgc-3’; HCF rev: 5’-catctcatgcatctgagagac-3’; PRC for: 5’-ggagagtgagctgc-3’; PRC rev: 5’-actctcttgctgacagtgc-3’; PERC/PDC-1β for: 5’-gtagtatcgtgatgagctgc-3’; PERC/PDC-1β rev: 5’-gtagaagctgagctgc-3’.

**Western blotting**

For western blots 50 mg total liver tissue of three different animals per time point (0, 24 or 48 h after injection with 50 µg T3/100 g body weight) were dissolved in SDS-PAGE gel running buffer,
Table 1 T3-regulated genes in livers of hypothyroid rats

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene name</th>
<th>Accession no.</th>
<th>Function</th>
<th>Fold increase</th>
<th>Verification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h 24 h 48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>EST (similar to cystathionine gamma-lyase (Cth))</td>
<td>AI427530</td>
<td>detoxification</td>
<td>0·5 0·2 0·2</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>EST (similar to alcohol dehydrogenase PAN2)</td>
<td>AI430962</td>
<td>metabolism</td>
<td>0·6 0·6 0·4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase P</td>
<td>AI327212</td>
<td>detoxification</td>
<td>0·5 0·5 0·4</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>EST (hypothetical protein MMT-7 gene)</td>
<td>AI414386</td>
<td>unclassified</td>
<td>1·4 1·7 3·1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
<td>AI323769</td>
<td>detoxification</td>
<td>1·3 1·6 2·6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to elongation of very long chain fatty acids-like 2)</td>
<td>AI448451</td>
<td>metabolism</td>
<td>1·5 2·4 4·7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribosomal protein L30</td>
<td>AI324671</td>
<td>translation</td>
<td>1·5 1·5 2·1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCAAT-binding transcription factor (Nfix)</td>
<td>AI528802</td>
<td>transcription</td>
<td>1·7 2·9 5·3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to brain protein 44-like (Brp44l))</td>
<td>AI414240</td>
<td>unclassified</td>
<td>1·5 3·0 4·8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ras-related YPT1 protein</td>
<td>AI528700</td>
<td>signal transduction</td>
<td>1·4 2·4 3·0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to N-myc downstream regulated 2 (Ndr2))</td>
<td>AI413204</td>
<td>signal transduction</td>
<td>2·1 3·4 5·1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multifunctional aminoacyl-tRNA synthetase</td>
<td>AI450063</td>
<td>translation</td>
<td>1·7 2·7 3·8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha-amyrase 2B precursor</td>
<td>AI325237</td>
<td>metabolism</td>
<td>1·8 2·8 4·0</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI451318</td>
<td>unclassified</td>
<td>1·8 3·9 4·3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Groucho protein GRG1-L (Tle 1)</td>
<td>AI324893</td>
<td>transcription</td>
<td>1·7 5·4 5·4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apolipoprotein AIV</td>
<td>AI326922</td>
<td>metabolism</td>
<td>2·2 11·7 14·5</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>EST (similar to calmodulin binding protein 3 (Strn 3))</td>
<td>AI426660</td>
<td>signal transduction</td>
<td>2·6 10·5 7·0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to karyogamy protein (KAR4))</td>
<td>AI429285</td>
<td>cell division</td>
<td>2·5 11·6 4·7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-cell Lymphoma 3-encoded protein (BCL3)</td>
<td>AI528891</td>
<td>transcription</td>
<td>3·5 5·0 3·2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to frizzled homolog 8 (FZD8))</td>
<td>AI385637</td>
<td>transcription</td>
<td>1·0 1·6 2·0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adenine nucleotide translocator 2</td>
<td>AI325068</td>
<td>metabolism</td>
<td>0·9 2·7 4·8</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial electron-transfer-flavoprotein, alpha polypeptide</td>
<td>AI325520</td>
<td>metabolism</td>
<td>1·1 1·9 2·6</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>Chaperonin 10</td>
<td>AI327085</td>
<td>protein folding</td>
<td>1·2 2·3 2·5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to signal recognition particle 54 kDa)</td>
<td>AI451616</td>
<td>protein targeting</td>
<td>1·3 4·3 4·9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI415331</td>
<td>unclassified</td>
<td>1·2 4·5 6·2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>EST (similar to myofibrillogenesis regulator MR-2)</td>
<td>AI430836</td>
<td>cell structure</td>
<td>1·1 2·6 2·5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI449611</td>
<td>unclassified</td>
<td>1·1 2·5 2·6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to apolipoprotein AV)</td>
<td>AI414038</td>
<td>metabolism</td>
<td>1·2 3·4 3·5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to steroid 5 alpha-reductase)</td>
<td>AI414607</td>
<td>metabolism</td>
<td>1·1 5·5 3·4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EST (similar to transport-secretion protein 2.2 (TTS-2.2))</td>
<td>AI415049</td>
<td>unclassified</td>
<td>1·2 4·4 3·6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxidative stress-inducible protein tyrosine phosphatase (PTPase)</td>
<td>AI325917</td>
<td>detoxification</td>
<td>1·4 6·0 4·2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,2-Trans-enoyl-CoA isomerase</td>
<td>AI323562</td>
<td>metabolism</td>
<td>0·9 2·6 2·9</td>
<td>S, N</td>
</tr>
<tr>
<td></td>
<td>c-Src kinase</td>
<td>AI324651</td>
<td>signal transduction</td>
<td>0·9 2·9 2·6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myelin basic protein</td>
<td>AI323506</td>
<td>cell structure</td>
<td>0·9 2·1 1·7</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>No.</th>
<th>Gene name</th>
<th>Accession no.</th>
<th>Function</th>
<th>Fold increase</th>
<th>Verification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>35</td>
<td>Epidermal growth factor receptor (Egfr)</td>
<td>AI427644</td>
<td>signal transduction</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>36</td>
<td>EST (similar to isocitrate dehydrogenase 1)</td>
<td>AI327112</td>
<td>metabolism</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>37</td>
<td>ATP synthase beta-chain</td>
<td>AI327309</td>
<td>metabolism</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>38</td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI449762</td>
<td>unclassified</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>39</td>
<td>EST (similar to zinc-finger protein (bcl-6))</td>
<td>AI528676</td>
<td>transcription</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>EST (similar to (predicted) yeast and worm proteins homolog)</td>
<td>AI325979</td>
<td>unclassified</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>41</td>
<td>Aldolase 1, A isoform</td>
<td>AI323970</td>
<td>metabolism</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>42</td>
<td>Farnesyl pyrophosphate synthase (Fpps)</td>
<td>AI450071</td>
<td>metabolism</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>43</td>
<td>EST (similar to neighbor of A-kinase anchoring protein 95)</td>
<td>AI448274</td>
<td>signal transduction</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>44</td>
<td>EST (similar to alpha/beta-hydrolases structure containing protein)</td>
<td>AI449506</td>
<td>metabolism</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>45</td>
<td>EST (similar to lipase)</td>
<td>AI448275</td>
<td>metabolism</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>46</td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI428422</td>
<td>unclassified</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>47</td>
<td>EST (no high similarity to any known gene product)</td>
<td>AI428535</td>
<td>unclassified</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>48</td>
<td>EST (similar to MHC region containing the Q region of class I)</td>
<td>AI414814</td>
<td>immunology</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>49</td>
<td>EST (similar to cytochrome P450)</td>
<td>AI426508</td>
<td>metabolism</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>50</td>
<td>EST (similar to hydroxyacid oxidase 3 (medium-chain))</td>
<td>AI325478</td>
<td>metabolism</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>51</td>
<td>Guanosine diphosphate (GDP) dissociation inhibitor 3</td>
<td>AI528719</td>
<td>metabolism</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>52</td>
<td>EST (similar to ubiquitin-conjugating enzyme E2I)</td>
<td>AI666653</td>
<td>protein degradation</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>53</td>
<td>EST (similar to neuron specific gene family member 1)</td>
<td>AI428539</td>
<td>signal transduction</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>54</td>
<td>EST (similar to ubiquitin specific protease 24)</td>
<td>AI414051</td>
<td>protein degradation</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>55</td>
<td>EST (similar to pantothenate kinase 1 (Pank1))</td>
<td>AI644488</td>
<td>metabolism</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>56</td>
<td>EST (similar to bromomain-containing protein 3)</td>
<td>AI428536</td>
<td>transcription</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>57</td>
<td>EST (similar to transcription factor BEN-beta (Gtf2ird1))</td>
<td>AI428491</td>
<td>transcription</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>58</td>
<td>Heat-responsive protein 12</td>
<td>AI893634</td>
<td>signal transduction</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>59</td>
<td>Alpha-tubulin isotype M</td>
<td>AI325223</td>
<td>cell structure</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>60</td>
<td>HMG-CoA synthase</td>
<td>AI324952</td>
<td>metabolism</td>
<td>0.4</td>
<td>1.1</td>
</tr>
<tr>
<td>61</td>
<td>EST (similar to CBP/p300-interacting transactivator)</td>
<td>AI449991</td>
<td>transcription</td>
<td>0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>62</td>
<td>NADH-ubiquinone oxidoreductase</td>
<td>AI451384</td>
<td>metabolism</td>
<td>0.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The expression ratios represent the mean of four independent experiments, with each gene spotted in duplicates. Gene names, GenBank accession numbers, gene functions and fold increase data are given. Fold increase data were calculated by comparing the 6, 24 and 48 h T3-treated signal intensities to the hypothyroid signal intensities (0 h) (see Result section). Selected genes were verified by sequencing (S), northern hybridisation (N) or quantitative real-time PCR (P).
boiled for 10 min at 95 °C and adjusted to an SDS-PAGE. Separated hepatic proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with a 1:1000 dilution of antibodies to Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), L-FABP (HyCult, Uden, The Netherlands), mGPDH (Weitzel et al. 2001a) or PGC-1α (Chemicon, Temecula, CA, USA) in 154 mM NaCl, 10 mM NaH₂PO₄, pH 7.5, 2.5% nonfat dry milk. Antibody binding was detected by carrying out secondary antibody incubations using peroxidase-conjugated anti first IgG antibodies (Dianova, Hamburg, Germany) diluted 1:5000. Secondary antibody was detected using the ECL system according to the manufacturer’s recommendation (Amersham Pharmacia).

### Results

#### Microarray analyses

Utilizing DNA microarray technology we investigated 4608 different genes to gain detailed information about T3-mediated gene expression patterns in rat liver in vivo. Total RNA from the livers of three animals per time point (0, 6, 24 and 48 h after administration of T3 in hypothyroid rats) was prepared and used for the labelling reaction and microarray hybridization. Microarray results of authenticated cDNAs were subsequently verified by northern blot, quantitative PCR and western blot experiments. Sixty-two of 4608 genes were shown to be reproducibly T3-responsive. Cluster analysis grouped these 62 differentially regulated genes into six well-defined expression profiles, which are summarized in Fig. 1.

#### Cluster analysis

The six observed clusters can be further subdivided into three functional groups. The first group exhibits a quick and steadily intensifying response (cluster 1 representing continuous down-regulation, while cluster 2 represents the reverse pattern, Figs 1 and 2). A second group displays a weak or even slightly negative response within the first 6 h followed by an increased expression after 48 h (cluster 3 and 5) and a third group that shows a distinct down-regulation after 6 h and is subsequently up-regulated (cluster 4 and 6) (Figs 1 and 2). In general, after 6 h approximately half of the genes were down- and half of the genes were up-regulated, whereas after 48 h only 3 of 62 are down-regulated in response to T3 (cluster 1, Fig. 1). Among the T3-responsive genes, which we have identified in our study, several genes have already been described to be T3 regulated, e.g. ANT2, myelin basic protein, ATP synthase subunit or apolipoprotein AIV (Dümmler et al. 1996, Miller et al. 2001, Clement et al. 2002, Flores-Morales et al. 2002). Thus, our screening protocol was successful in identifying differentially T3-regulated genes.

#### Verification of microarray hybridization data

Representative examples of differentially regulated genes from each of the six different gene profile clusters were selected for further characterization by either northern blot experiments or quantitative
Expression patterns in T3-treated rats

J M WEITZEL, S HAMANN and others

Journal of Molecular Endocrinology

www.endocrinology.org

Cluster #

hours after induction

 Cluster 1

Cluster 2

Cluster 3

Cluster 4

Cluster 5

Cluster 6

log2 scale

-4 -2 -1 0 1 2 4

repression induction

Downloaded from Bioscientifica.com at 03/03/2022 08:11:24AM via free access
Average Cluster Profiles

Cluster Verification

Cluster 1
(n=3)
Cth

Cluster 2
(n=16)
Apo AIV

Cluster 3
(n=15)
enoxy-CoA isomerase

Cluster 4
(n=3)
ATP synth.

Cluster 5
(n=21)
EST Al414814

Cluster 6
(n=4)
HMG-CoA synthase
real-time PCR. For northern blot analysis, identical amounts of total RNA (combined from three different animals per time point) were electrophoresed and visualized by staining with ethidium bromide (data not shown). Additional quality control was performed by hybridization with the ‘housekeeping’ gene ubiquitin (data not shown). In summary, we were able to reproduce the cluster profiles which have been detected by microarray hybridization using northern blot hybridization and quantitative real-time PCR (Fig. 2).

**Transient transfection analysis of late T3-induced genes**

Since at least 36 out of the 62 differently regulated genes (58%) were not significantly regulated within the first 24 h (clusters 3 and 5), we tested the hypothesis that eight gene promoters of six late-induced genes (e.g. ANT2 (Table 1, No. 21) (Scarpulla et al. 1986, Luciakova & Nelson 1992, Weitzel et al. 2001b) are directly regulated via a TRE. As indicated in Table 2 (and data not shown) we were unable to detect a TRE in these gene promoters by transient transfection experiment in human hepatocarcinoma HepG2 cells. As positive control we used the well-characterized TRE-containing promoter B of the rat mitochondrial glycerol-3-phosphate dehydrogenase gene (an early-induced gene (Dümmler et al. 1996)) under identical experimental conditions (Weitzel et al. 2001a).

**Candidates for an intermediate factor of T3 action**

Numerous distinguishable T3-mediated expression patterns, together with a lack of TREs in late-induced gene promoters, suggested multiple pathways of T3-mediated modulation of gene expression which probably includes the action of an intermediate factor(s). In this study we analyzed: (i) rather ubiquitously expressed transcription factors, (ii) ligand-dependent transcription factors and (iii) coactivators of the PGC-1 family as candidates for an intermediate factor of T3 action, which were either undetectable by microarray hybridisation or not present on the array (data not shown). In order to analyze the gene expression levels 0, 6, 24 and 48 h after the administration of T3 we performed quantitative real-time PCR, as described above.

We observed no significant T3-regulation of the transcription factors CREB, HCF, HNF-4α, Sp1 and YY1, but discerned a regulation of the mitochondrial transcription factor A (mtTFA) (Table 3). Analyses of the ligand-dependent transcription factors PPARs (peroxisome proliferator-activated receptors) revealed a different T3-mediated regulation of the three known isoforms. Whereas PPARα was late-induced by T3, PPARβ was not induced and PPARγ was down-regulated within the first 6 h and remains reduced for at least 48 h after the administration of T3. The coactivators PRC (Andersson & Scarpulla 2001) and PERC/PGC-1β (PGC-1-related estrogen receptor coactivator (Kressler et al. 2002, Lin et al. 2002)) are regulated within the first 6 h after T3 treatment and the abundance remains high for at least 48 h. The T3-mediated expression patterns of transcription factors and coactivators are summarized in Table 3.

**Alteration of protein concentrations in response to T3**

In order to verify that an increased concentration of mRNA molecules (which we measured by microarray experiments, northern analyses and quantitative PCR) also leads to increased protein abundance, we performed an analysis by western blot. Western blot experiments were conducted using identical amounts of total protein extract from livers of untreated and T3-treated rats (0, 24 and 48 h after the administration of T3) that were loaded onto an SDS-PAGE and blotted according to standard protocols.

Not surprisingly, protein quantities were not altered when the amount of corresponding mRNA
did not change (e.g. for Sp1, Fig. 3A). When mRNA abundance did not shift within the first 24 h after T3-treatment (e.g. liver fatty acid binding protein (L-FABP), apolipoprotein AI and apolipoprotein AIV), we did not observe alteration in protein concentrations within 48 h either (Fig. 3A and data not shown). In contrast, those genes which were induced within the first 6 h also display increased protein abundance after 24 h which is more pronounced after 48 h (e.g. mGPDH and PGC-1α, Fig. 3B).

### Discussion

T3 has a profound influence in mitochondrial biogenesis and metabolic balance. Consistent with this long known observation, DNA microarray analysis identified numerous genes implicated in metabolic pathways to be regulated by T3 (Feng et al. 2000, Miller et al. 2001, Weitzel et al. 2001b, Clement et al. 2002, Flores-Morales et al. 2002, Wood et al. 2002). Our microarray analysis detected changes in the expression of ANT2, apolipoprotein A1V, HMG-CoA synthase and ATP synthase β subunit, all four cases representing examples of T3-regulated genes that are associated with metabolic pathways. However, the majority of the differentially regulated genes are not primarily involved in metabolic functions; they are associated

### Table 3 Differentially regulated candidate intermediate factors in T3-treated rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA levels (fold increase)</th>
<th>Protein levels (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>1) transcription factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CREB</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>HCF</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>mtTFA</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Sp1</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>YY1</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>2) peroxisome proliferator-activated receptors (PPARs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARα</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>PPARβ</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>PPARγ</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3) PPARγ coactivator 1 (PGC-1) family of coactivators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGC-1α</td>
<td>13.0</td>
<td>9.4</td>
</tr>
<tr>
<td>PRC</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>PERC/PGC-1β</td>
<td>5.0</td>
<td>6.9</td>
</tr>
</tbody>
</table>

Fold increase data from at least three quantitative real-time PCR experiments or western blot experiments are given. The values were calculated by comparing the 6, 24 and 48 h hyperthyroid signal intensities to the hypothyroid signal intensities (0 h) within the same experiment. n.d., not done.
with a wide variety of cellular pathways, encompassing translation, protein turnover, cell structure and apoptosis-associated proteins (Table 1).

Cluster analysis divided the 62 differentially regulated genes into six expression profiles (Figs 1 and 2). Remarkably, the vast majority of T3-responsive genes were found to be represented by clusters 2, 3 and 5 (52 of 62 genes) (Fig. 1). In contrast, cluster 1 (3 of 62 genes), cluster 4 (3/62) and cluster 6 (4/62) apparently depict rarely occurring patterns.

It is interesting to compare the very similar T3-dependent gene expression patterns in rat liver and *Xenopus laevis* metamorphosis. Metamorphosis in *Xenopus laevis* is characterized by a non-functional thyroid gland in embryos, which is converted into a functional (thyroid hormone-secreting) organ during larval development – a classical T3-dependent differentiation model (Shi et al. 1996, Berry et al. 1998). Among the differentially T3-regulated genes during metamorphosis, Shi and co-workers described a biphasic expression pattern for the neural-specific tubulin β subunit in *Xenopus* (Denver et al. 1997) and, noteworthy, we identified a highly similar biphasic expression pattern for an α-tubulin isoform in rat liver (cluster 6, Table 1, No. 59). This suggests an evolutionarily conserved principle of T3 activation of homologous genes in amphibian and mammals.

Numerous distinguishable T3-mediated expression patterns suggest multiple pathways of T3-mediated modulation of gene expression. This is supported by recent findings of gene-specific effects of TRα, TRβ and SRC-1 on T3-regulated gene expression (Flores-Morales et al. 2002, Takeuchi et al. 2002, Sadow et al. 2003), although temporal expression patterns have not been analyzed in these studies. Furthermore, some genes are known to be endogenously regulated by T3 in *vivo*; however, a thyroid hormone response element (TRE) has not yet been identified within their regulatory elements (Table 2). Since the great majority of TREs are located several hundred base pairs upstream of the transcriptional start site (Yen 2001, Weitzel et al. 2003) it is unlikely that a TRE is located upstream of the investigated promoter sequences; however we cannot completely exclude this possibility. Moreover, all these genes are regulated late (~12–24 h) in response to T3. This leads to the assumption that there might be an intermediate factor of T3 action, modulating the expression of late-induced, no TRE-containing genes. Since the synthesis of this intermediate factor requires time, the T3 response of the late-induced target genes exhibits a latency period. In our previous study, we suggested the transcription factor NRF-1 and the coactivator PGC-1α as candidate factors for such an intermediate factor. Unfortunately, a common sequence motif in late-induced genes (e.g. a common NRF-1 site) has not yet been described in late-induced gene promoters (Weitzel et al. 2003).

We therefore tested the hypothesis whether other transcription factors might serve as intermediate factors. However, neither Sp1 nor YY1, CREB, HCF or HNF-4α (either transcriptional regulators of late-induced genes or PGC-1 interaction partners (Evans & Scarpulla 1989, Basu et al. 1993, Seelan & Grossman 1997, Tagami et al. 1999, Zaid et al. 1999, Yoon et al. 2001, Lin et al. 2002)) have been shown to be regulated by T3 (Table 3). In contrast, mtTFA, the major transcription factor of the mitochondrial genome, is up-regulated within 48 h after administration of T3, in agreement with previous studies (Garstka et al. 1994).

Many of the detected target genes are involved in lipid metabolism (e.g. apolipoprotein AIV, enoyl-CoA isomerase, HMG-CoA synthase, L-FABP, sterol carrier protein 2 (SCP2)) and are known to be regulated by PPARs (Hsu et al. 2001, Poirier et al. 2001). Furthermore, the concentration of activated fatty acids is increased in hyperthyroidism (Müller & Seitz 1981). We therefore evaluated the hypothesis that PPAR transcription factors might serve as intermediate factors. Surprisingly, the observed expression pattern of PPAR isoforms α, β and γ exhibits a completely different profile in response to T3 (Table 3). Solely PPARγ was (down-)regulated within 6 h and remains down-regulated for at least 48 h, a very unusual expression profile (see cluster 1, Fig. 1). Since PPARγ has also been described as a differentiation factor for adipogenesis rather than a regulator of fatty acid oxidation (Lowell 1999), down-regulation may primarily be involved in differentiation processes in response to T3.

Finally, we examined whether expression of the coactivators of the PGC-1 family is regulated by T3. We previously described PGC-1α to be dramatically regulated by T3 in rat liver within 6 h (Weitzel et al. 2001b). Very similar results have now
have been reported for PGC-1 related coactivator (PRC) and PERC/PGC-1β. Thus, the PGC-1 family members of coactivators are highly attractive candidates for mediating the late induction pattern (Table 3 and Fig. 3).

Investigations of the steady-state level of mRNA concentrations are hampered by the question of whether modulation of the mRNA concentration also reflects a modulation of the protein concentration. Hence we tested various candidate proteins, where the corresponding mRNA is: (i) not regulated by T3, (ii) late regulated by T3 and (iii) early regulated by T3. We observed that a change in protein concentration did not occur within 48 h, when there is no significant alteration in the mRNA within the first 24 h (Fig. 3A and Table 3). However, in those cases where we observed an increase in the mRNA abundance within 6 h after T3-treatment (early induction), the protein concentration was also altered after 24 h and the change was even more pronounced 48 h after administration of T3 (Fig. 3B and Table 3).

In summary, our data reveal a complex expression pattern in response to T3 in rat liver in vivo, suggesting various signal transduction pathways of T3 action. Aside the ‘classical’ pathway of T3-mediated gene regulation by thyroid hormone receptor binding to TREs, an additional pathway appears to be mediated by transcription factors like NRF-1 and PPARγ (but not CREB, HCF, HNF-4α, NRF-2, Sp1, YY1, PPARα, PPARβ) and coactivators like the PGC-1 family of coactivators. It has been shown that ectopic expression of PGC-1α mimics T3-mediated effects (Wu et al. 1999, Knutti & Kraalli 2001, Yoon et al. 2001, Puigserver & Spiegelman 2003). It will be interesting to address the question of whether signal pathways are lost after depletion of these factors, as soon as suitable knockout models become available.

Acknowledgements

We are indebted to Britta Schwarzlof for help with the LightCycler, and to Richard C Scarpulla (Northwestern University, Chicago, USA), Jerome F Strauss III (University of Pennsylvania, USA) and Rudolf J Wiesner (University of Köln, Germany) for kind gifts of plasmid DNA. This work was supported by grants from the Hamburgische Wissenschaftliche Stiftung and the Deutsche Forschungsgemeinschaft (WE 2458/3–1) to J M W.

References


Expression patterns in T3-treated rats · J M WEITZEL, S HAMANN and others

Thyroid hormone activates transcription from the promoter
regions of some human nuclear-encoded genes of the oxidative
phosphorylation system. *Molecular and Cellular Endocrinology* **128**
69–75.

Peroxisome proliferator-activated receptor gamma coactivator 1
beta (PGC-1 beta), a novel PGC-1-related transcription
coactivator associated with host cell factor. *Journal of Biological
Chemistry* **277** 1645–1648.

Lovell BB 1999 PPARgamma: an essential regulator of adipogenesis

Luciakova K & Nelson BD 1992 Transcript levels for nuclear-
encoded mammalian mitochondrial respiratory-chain components
are regulated by thyroid hormone in an uncoordinated fashion.

Miller LD, Park KS, Guo QM, Akharouf NW, Malek RL, Lee NH,
Liu ET & Cheng SY 2001 Silencing of Wnt signaling and
activation of multiple metabolic pathways in response to thyroid
hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21**
6626–6639.

Müller MJ & Seitz HJ 1981 Starvation-induced changes of hepatic
protein kinase C in rat rapidly growing and replicating
hepatocytes. *Journal of Biological Chemistry* **256**
1370–1379.

Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C,
Luciakova K & Nelson BD 1992 Transcript levels for nuclear-
encoded mammalian mitochondrial respiratory-chain components
are regulated by thyroid hormone in an uncoordinated fashion.

Miller LD, Park KS, Guo QM, Akharouf NW, Malek RL, Lee NH,
Liu ET & Cheng SY 2001 Silencing of Wnt signaling and
activation of multiple metabolic pathways in response to thyroid
hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21**
6626–6639.

Müller MJ & Seitz HJ 1981 Starvation-induced changes of hepatic
protein kinase C in rat rapidly growing and replicating
hepatocytes. *Journal of Biological Chemistry* **256**
1370–1379.

Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C,
Luciakova K & Nelson BD 1992 Transcript levels for nuclear-
encoded mammalian mitochondrial respiratory-chain components
are regulated by thyroid hormone in an uncoordinated fashion.

Miller LD, Park KS, Guo QM, Akharouf NW, Malek RL, Lee NH,
Liu ET & Cheng SY 2001 Silencing of Wnt signaling and
activation of multiple metabolic pathways in response to thyroid
hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21**
6626–6639.

Müller MJ & Seitz HJ 1981 Starvation-induced changes of hepatic
protein kinase C in rat rapidly growing and replicating
hepatocytes. *Journal of Biological Chemistry* **256**
1370–1379.

Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C,
Luciakova K & Nelson BD 1992 Transcript levels for nuclear-
encoded mammalian mitochondrial respiratory-chain components
are regulated by thyroid hormone in an uncoordinated fashion.

Miller LD, Park KS, Guo QM, Akharouf NW, Malek RL, Lee NH,
Liu ET & Cheng SY 2001 Silencing of Wnt signaling and
activation of multiple metabolic pathways in response to thyroid
hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21**
6626–6639.

Müller MJ & Seitz HJ 1981 Starvation-induced changes of hepatic
protein kinase C in rat rapidly growing and replicating
hepatocytes. *Journal of Biological Chemistry* **256**
1370–1379.

Poirier H, Niot I, Monnot MC, Braissant O, Meunier-Durmort C,
Luciakova K & Nelson BD 1992 Transcript levels for nuclear-
encoded mammalian mitochondrial respiratory-chain components
are regulated by thyroid hormone in an uncoordinated fashion.

Miller LD, Park KS, Guo QM, Akharouf NW, Malek RL, Lee NH,
Liu ET & Cheng SY 2001 Silencing of Wnt signaling and
activation of multiple metabolic pathways in response to thyroid
hormone-stimulated cell proliferation. *Molecular and Cellular Biology* **21**
6626–6639.

Müller MJ & Seitz HJ 1981 Starvation-induced changes of hepatic
protein kinase C in rat rapidly growing and replicating
hepatocytes. *Journal of Biological Chemistry* **256**
1370–1379.