Differential modulation of expression of the two acylphosphatase isoenzymes by thyroid hormone

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The modulation of expression of the skeletal muscle and erythrocyte acylphosphatase isoenzymes by thyroid hormone has been investigated. Our results indicate a differential regulation of the two enzymic isoforms by tri-iodothyronine (T_3) in K562 cells in culture: an increase in the specific mRNA during T_3-stimulation is shown only for the skeletal muscle isoenzyme. A fast and transient T_3 induction of the accumulation of the specific mRNA can be observed, reaching a maximum 8 h after hormone treatment and then rapidly decreasing almost to the steady-state level after 24 h. A nuclear run-on assay was performed to explore the mechanisms of this regulation. These studies indicate that T_3 induction of skeletal muscle acylphosphatase mRNA is due, at least in part, to a fast and transient increase in the rate of gene transcription, within 4 h after hormone administration. A very rapid decrease is then observed within a further 2 h. T_3-dependent accumulation of the mRNA for the skeletal muscle acylphosphatase requires ongoing protein synthesis, as confirmed by inhibition with cycloheximide or puromycin. These findings indicate that the transcriptional regulation of the gene may be indirect.

INTRODUCTION

Acylphosphatase (EC 3.6.1.7) is a cytosolic hydrolase that is widely distributed in animal tissues and is catalytically active on compounds with a carboxyl-phosphate bond. Among natural substrates, 3-phosphoglyceroyl phosphate [1], carbamoyl phosphate [2] and succinoyl phosphate [3] are hydrolysed by the enzyme. Two isoenzymic forms of acylphosphatase are known, named skeletal muscle and erythrocyte acylphosphatase respectively because of their major location, although they are present in virtually all animal tissues. Both enzymes are 98 amino acids in length, and the sequences show significant identity (about 60%). They exhibit similar substrate specificity and kinetic properties, except that the erythrocyte isoenzyme appears to have higher catalytic activity. It has been demonstrated that the muscle isoenzyme is able to hydrolyse the carboxyl-phosphate bond formed in membrane pumps such as the Na^+/K^+-ATPase of the plasma membrane and the Ca^{2+}/Mg^{2+}-ATPase of the sarcoplasmic reticulum [4,5]. The erythrocyte isoenzyme stimulates Ca^{2+}-ATPase activity in red blood cell plasma membranes, lowering Ca^{2+} transport and thus acting as a pump inhibitor [6]. Furthermore, both acylphosphatase isoenzymes, when added exogenously to permeabilized human platelets, are able to modify the levels of phosphoinositides in such cells, although they are not able to hydrolyse these compounds *in vitro* [7].

The skeletal muscle isoenzyme could be considered as a short-lived muscle-specific protein and appears to be linked to myogenic differentiation [8]. The increase in the enzyme during *in vitro* differentiation of L6 rat myoblasts is very similar to that of creatine kinase, a specific muscle isoenzyme, and shows a similar time course and extent. Its increase in myotubes appears to be accompanied by activation of its breakdown, as indicated by determination of the half-life of the enzyme. Acylphosphatase does not contain sequences rich in proline, aspartic/glutamic acid and serine/threonine, commonly called PEST sequences, but is a very small, basic and hence strongly positively charged protein, which could explain its inclusion in the 2.5% of cell proteins that are described as short-lived proteins.

As regards physiological function, since all the substrates of acylphosphatase are 'high-energy' phosphorylated compounds, these enzymes could be involved in energy metabolism, and it is possible that changes in their concentration may provide a biochemical basis for the regulation of energy expenditure. It has been proposed that acylphosphatase could hasten glycolysis in response to the hydrolysis of glycerol 3-phosphate, at the expense of ATP formation, with an uncoupling effect similar to that elicited experimentally by arsenate [9]. On the other hand, hydrolysis of the Ca^{2+}-ATPase aspartyl phosphate intermediate could suggest possible involvement in the control of this active transport system. The possible role of the enzyme in energy metabolism, suggested also by the observation that acylphosphatase could be involved in the first step of hibernation awakening [10], made a correlation possible between thyroid hormone action and acylphosphatase levels. Studies on erythrocytes from hyperthyroid patients and normal controls showed a correlation between the increase in acylphosphatase content and hyperthyroidism [11]. The effect of tri-iodothyronine (T_3) on Na^+/K^+-ATPase activity in K562 human erythroleukaemia cells was studied to investigate why erythrocyte sodium pump activity is decreased in hyperthyroidism [12]. These observations, together with the above-mentioned involvement in myogenic differentiation, led us to consider the fascinating hypothesis of gene control by thyroid hormone of the two isoforms of acylphosphatase.

In this paper, using a human erythroleukaemia cell line as a cell culture system that is responsive to T_3, we demonstrate that only the expression of skeletal muscle acylphosphatase is controlled by thyroid hormone at a transcriptional level and that ongoing protein synthesis is required for the stimulation of gene transcription by T_3. On the other hand, the lack of the same

Abbreviations used: T_3, 3,5,3′-tri-iodo-L-thyronine; T_4, 3,5,3′,5′-tetra-iodo-L-thyronine; FCS, fetal calf serum; PTPase, phosphotyrosine protein phosphatase; SSC, standard saline citrate buffer, GAD, glyceraldehyde-3-phosphate dehydrogenase.

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hormonal control for the erythrocyte isoenzyme supports our previous hypothesis of possible divergent roles *in vivo* for these two isoenzymes.

MATERIALS AND METHODS

Materials

AG1-X8 ion-exchange resin was purchased from Bio-Rad; Dulbecco's modified Eagle's medium and fetal calf serum (FCS) were from GIBCO Laboratories; Taq DNA polymerase, T4 polynucleotide kinase and restriction enzymes were from Promega; Sequenase 2.0 was from USBC; cDNA construction library kit was from Amersham; oligonucleotides were from Pharmacia; [α-^{32}P]dATP, [γ-^{32}P]dATP and [α-^{32}P]UTP (all 3000 Ci/mmol) were from NEN. T_3, puromycin and cycloheximide were purchased from Sigma; RNase-free DNase and protease K were from Boehringer Mannheim; Hybond N$^+$ nylon membrane was from Amersham; T_3/T_4 ELISA test was from Boehringer Mannheim. Other chemicals were from Sigma. Bluescript SK plasmid vector was from Stratagene.

cDNA isolation

For the erythrocyte isoform, a 500 ng DNA sample from a human placental cDNA library in λgt11 phage vector was used as a template in the PCR, primed with two degenerate oligonucleotides for 40 temperature cycles. Each cycle consisted of 1 min each at 95 °C, 42 °C and 72 °C in an MJ Research Minicycler. The PCR buffer was 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 15 mM Tris/HCl, pH 8.4. The sequences of the two oligonucleotides are 5′-GTNGAC(T)TAC(T)GAG(A)ATA-(CT)TTC(T)GG and 5′-TTG(A)AAG(A)TTG(A)TTG(A)CTC-(T)TTC(T)CA, which correspond to amino acids 9–15 (Val-Asp-Tyr-Glu-Ile-Phe-Gly) and 79–85 (Asn-Phe-Asn-Asn-Glu-Lys-Val) of the human erythrocyte acylphosphatase [13].

For the muscle isoform, screening of a λgt11 human heart cDNA library was performed with standard methods, using as probes two degenerate oligonucleotides deduced from the protein sequence. The sequences of the two oligonucleotides are 5′-ATGTAC(T)ACNGAA(G)GAT(C)GAA(G)GC and 5′-ACT-(C)TTGTCT(C)TCNGGNCCCTG, which correspond to amino acids 24–30 (Met-Tyr-Thr-Glu-Asp-Glu-Ala) and 52–58 (Gln-Gly-Pro-Glu-Asp-Lys-Val) respectively [14]. Probes were labelled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 nucleotide kinase.

Cell culture

K562 cells [15] were routinely cultured in RPMI 1640 medium (Gibco BRL), supplemented with 10% (v/v) total FCS and incubated in 90 mm Petri dishes in an atmosphere of 95% air and 5% CO_2. T_3-depleted FCS was obtained by AG1-X8 ion-exchange treatment using the Samuel procedure [16]. K562 cells were maintained in T_3-depleted FCS medium for at least 8 days before T_3 stimulation. Hormone was dissolved in DMSO (1 mg/ml) and diluted 1:1000 in the culture medium. Cycloheximide and puromycin were dissolved in DMSO (32 and 200 mM respectively) and added to the culture medium, diluted 1:1000, 2 h before the addition of T_3. In all cases control cultures received an equal volume of solvent alone.

Protein determination and enzyme immunoassay

Protein determination was performed on cell lysates in glycyl/glycine (50 mM), pH 6.25, according to Lowry et al. [17]. Human skeletal muscle acylphosphatase determinations were carried out by a non-competitive sandwich ELISA performed using microtitre enzyme immunoassay plates coated with anti-(human skeletal muscle acylphosphatase) polyclonal antibodies affinity purified from rabbit antisera. After washing cycles, dilutions of standard and samples in the assay buffer (20 mM Tris/HCl, 137 mM NaCl, pH 7.6, containing 1% BSA and 0.05% Tween 20) were added to the wells and incubated overnight at 4 °C. After washing, the appropriate dilution of the same antibody conjugated to horseradish peroxidase was incubated in the wells for 3 h at room temperature. Quantification of immunocomplexes was determined by measuring peroxidase activity using *o*-phenylenediamine as substrate. The basal level of the enzyme and its expression were assessed by using standard solutions of human acylphosphatase. The linear range of the assay was 0.1–2 ng/ml. Cell lysates, depleted of the enzyme by immunoprecipitation and supplemented with standard, showed a linear and reproducible concentration–absorbance relationship, very similar to that obtained with standard in assay buffer.

DNA probes

The cDNA probes for human actin [18] and rat glyceraldehyde-3-phosphate dehydrogenase (GAD) [19] were generously provided by Dr. E. Meacci and Dr. G. Bensi respectively. cDNA coding for human low-M_r phosphotyrosine protein phosphatase (PTPase) was previously isolated in our laboratory [20].

Northern blot analysis

RNA was isolated from cells by the guanidinium thiocyanate/phenol/chloroform method [21]. Total RNA was quantified spectrophotometrically, and subjected to electrophoresis in a 1.2% agarose gel in 23 mM Mops and 15% formaldehyde. After electrophoresis, RNAs were transferred to a Hybond N$^+$ nylon membrane (Amersham), prehybridized for 4 h and hybridized for 16 h in 4 × standard saline citrate (SSC) (150 mM NaCl, 15 mM sodium citrate), 5 × Denhart's solution, 0.1% SDS and 50% formamide at 42 °C. Three washes were performed in 4 × SSC and 0.1% SDS at 65 °C. Filters were subjected to autoradiography at −70 °C with Kodak XAR 5 film and intensifying screens. The exposed films were scanned in a Cybertech Image analyser.

Isolation of nuclei and transcription run-on assay

Nuclei were isolated from K562 cells in culture by modifiying a previously described procedure [22]. Briefly, 5×10^7 cells were harvested, washed twice in PBS (100 mM NaCl, 150 mM NaH_2PO_4, pH 7.5) and treated for 5 min in 3 ml of lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4, 0.5% Nonidet P-40). The suspension was centrifuged at 1000 *g* for 10 s and pellets were resuspended in an equal volume of nuclei storage buffer (40% glycerol, 50 mM Tris/HCl, pH 8, 5 mM $MgCl_2$, 0.1 mM EDTA) and stored, until needed, at −80 °C.

The *in vitro* transcription assay was a modification of previously described procedures [23]. A 100 μl portion of nuclei was mixed with an equal volume of reaction buffer [50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 5 mM dithiothreitol, 150 mM KCl, 10% glycerol, 7 mM CTP, 7 mM ATP, 7 mM GTP, 8 μM UTP and 1 mCi of [^{32}P]UTP (3000 Ci/mmol)]. The reaction was incubated at 26 °C for 20 min and terminated by the addition of 10 μg of RNase-free DNase. After 10 min at room temperature, proteinase K was added at a final concentration of 1 mg/ml. The incubation was continued for an additional 2 h at 45 °C with continuous agitation. The solution was extracted with phenol/chloroform (1:1, v/v) and the labelled transcripts were pre-

cipitated with trichloroacetic acid and resuspended for 10 min at 60 °C in 10 mM Tris/HCl and 1 mM EDTA with 0.1 % SDS. Slot-blot filters for the nuclear transcription assay were prepared as follows. DNA (2 μg) was denatured by boiling at 100 °C for 5 min and quickly chilled on ice. After the addition of a volume of 20 × SSC the DNA solution was 'slotted' on a Hybond N^+ nylon membrane (Amersham) as recommended by the manufacturer. The filter-bound DNA was prehybridized for 6 h at 65 °C in a solution containing 50 % formamide, 6 × SSC/0.1 % SDS and 5 × Denhart's solution. The labelled transcripts were added and hybridization was continued at 42 °C for 72 h. The filters were washed twice at room temperature in 6 × SSC/0.1 % SDS; twice at 65 °C in 2 × SSC/0.1 % SDS; and twice at 65 °C in 0.2 × SSC/0.1 % SDS. Autoradiography and quantification were performed in a Phosphor Imager (Molecular Dynamics).

RESULTS

Cloning of the cDNAs for human acylphosphatases

In order to isolate the cDNAs coding for the two different isoforms of human acylphosphatase, two different strategies were followed. For the erythrocyte form, two degenerate oligonucleotides were synthesized, based on the primary structure of the protein [13], corresponding to amino acids 9–15 and 79–85 respectively. A 500 ng portion of DNA from a λgt11 human placental cDNA library was used as a template in a PCR using the two degenerate oligonucleotides. The PCR product, of the predicted length of about 230 bp, was subcloned in the Bluescript SK vector. DNA sequence analysis of both strands by the Sanger method shows that this product corresponds to the desired erythrocyte acylphosphatase cDNA fragment (Figure 1). For the skeletal muscle isoform, a human heart cDNA library was screened using three degenerate oligonucleotides, synthesized on the basis of the protein sequence [14]. Few positive clones were isolated and the longest was about 520 bases in length. This sequence was subcloned in the Bluescript SK vector; analysis of both strands of cDNA by the Sanger method revealed that this clone spans the sequence coding for the last 78 amino acids and about 280 bases of the 3′ non-translated sequence. The 237-base-long coding sequence (Figure 1), obtained by PCR amplification, was used as probe in all experiments.

Alignment of the two DNA sequences coding for the two different isoforms shows only 62 % identity (Figure 1). As expected with such low identity, no cross-hybridization can be observed between the two probes even in medium-stringency conditions of hybridization (6 × SSC at 65 °C). These portions of the cDNA for human erythrocyte and skeletal muscle acylphosphatases were used as probes in Northern blot experiments, in high-stringency conditions. Each probe identified a specific mRNA of about 1400 bases in length. In Figure 2 a Northern blot of total RNA from K562 cells using the two probes is presented, showing that the specific mRNA for the erythrocyte form is more abundant (about 6-fold, as determined by densitometric analysis) in this cell line than that for the muscle form.

In order to confirm that there was no cross-hybridization between the two probes, in some experiments filters independently hybridized with the two cDNAs were exposed first after mild (6 × SSC) washing and again after high-stringency (0.5 × SSC) washing; no differences could be observed between the two treatments (results not shown).

Differential modulation of expression of the two isoforms by T_3

K562 cells respond to physiological concentrations of T_3 and T_4 when cultured with thyroid hormone-depleted medium that is supplemented with thyroidectomized-calf serum [12,15]. In the present study an alternative method to the use of this special serum, as described by Samuel et al. [16], was employed. With our method, euthyroid calf serum was treated with AG1-X8 ion-exchange resin, which completely removed thyroid hormones from FCS, while only minimally altering the low-M_r anion components and protein content of the serum. Evaluation of

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ERYT.AP   9-ValAspTyrGluIlePheGlyLysValGlnGlyValPhePheArgLysHisThrGlnAlaGluGlyLysLysLeuGly-34
            GTGGATTATGAAATTTTTGGGAAGGTGCAAAGGGTGTTTTTCCGTAAGCATACTCAGGCTGAGGGTAAAAAGCTGGGA-112
MUSC.AP                                                 TGCTTCAGAATGTATACAGAAGATGAAGCTAGGAAAATAGGA-42
                                                      21-CysPheArgMetTyrThrGluAspGluAlaArgLysIleGly-34

ERYT.AP  35-LeuValGlyTrpValGlnAsnThrAspArgGlyThrValGlnGlyGlnLeuGlnGlyProIleSerLysValArgHis-60
        113-TTGGTAGGCTGGGTCCAGAACACTGACCGGGGCACAGTGCAAGGACAATTGCAAGGTCCAATCTCCAAGGTGCGTCAT-188
MUSC.AP  43-GTGGTTGGCTGGGTGAAGAATACAAGCAAAGGCACCGTGACAGGCCAAGTGCAGGGGCCAGAAGACAAAGTCAATTCC-111
         35-ValValGlyTrpValLysAsnThrSerLysGlyThrValThrGlyGlnValGlnGlyProGluAspLysValAspSer-60

ERYT.AP  61-MetGlnGluTrpLeuGluThrArgGlySerProLysSerHisIleAspLysAlaAsnPheAsnAsnGluLysVal-85
        189-ATGCAGGAATGGCTTGAAACAAGAGGAAGTCCTAAATCACACATCGACAAAGCAAACTTCAACAATGAAAAAGTC-264
MUSC.AP 112-ATGAAGTCCTGGCTGAGCAAGGTTGGAAGCCCTAGTTCTCGCATTGACCGCACAAACTTTTCTAATGAAAAAACCATC-187
         61-MetLysSerTrpLeuSerLysValGlySerProSerSerArgIleAspArgThrAsnPheSerAsnGluLysThrIle-86

MUSC.AP 188-TCTAAGCTTGAATACTCTAATTTTAGTATTAGATACTAA-226
         87-SerLysLeuGluTyrSerAsnPheSerIleArgTyrcooH-98
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Figure 1 cDNA sequence alignment of erythrocyte and skeletal muscle acylphosphatases

Sequence alignment was performed with PC/GENE software (Intelligenetics, Inc.). Amino acid translation of the two cDNAs is shown. Numbers are related to the positions of amino acids in the native protein sequences. Vertical bars indicate identical bases. ERYT.AP, erythrocyte acylphosphatase; MUSC.AP, muscle acylphosphatase.

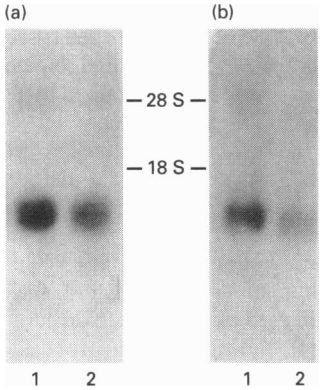

Figure 2 Identification of acylphosphatase mRNAs

Northern blot analysis was performed using the erythrocyte (**a**) or the skeletal muscle (**b**) acylphosphatase cDNA probes. Samples of 20 μg (lanes 1) or 3.5 μg (lanes 2) of total RNA purified from K562 cells were used in both hybridizations. Autoradiography was for 4 days (**a**) or for 8 days (**b**). Approximate positions of 28 S and 18 S rRNAs are indicated. This result is representative of three independent experiments.

Table 1 Effects of T_3 on the accumulation of the two acylphosphatase isoenzyme mRNAs

K562 cells were treated for at least 8 days in T_3-depleted medium and then 1 μg/ml T_3 was added. Northern blot analysis of total RNA (20 μg) was performed at different times as indicated, using both acylphosphatase isoenzyme cDNA probes. Results were quantified by densitometric scanning with Cybertech CS-1 Image Analysis Software. Each value, expressed as a percentage of the control, was normalized to signals obtained using a human actin cDNA as probe on the same filters. The results are means ± S.D. of three independent measurements.

	mRNA accumulation (% of control)			
Isoenzyme source	Control	5 h	8 h	24 h
Skeletal muscle	100	93 ± 9	785 ± 119	232 ± 35
Erythrocyte	100	111 ± 21	98 ± 20	107 ± 17

thyroid hormones with an ELISA test confirmed that the treatment removed more than 99% of T_4 and T_3 from fetal calf serum (results not shown).

To study the possible T_3-dependent regulation of acylphosphatase mRNA levels, K562 cells were maintained in culture for at least 8 days in a medium depleted of thyroid hormones. The erythroblasts, grown to a relatively high density (about 1.5×10^6 cells/ml), were transferred to fresh hormone-depleted FCS medium and supplemented with 1 μg/ml T_3 after 4 h. We performed a time course experiment, recovering samples 5, 8 and 24 h after hormone addition. Northern blot analysis of total cellular RNA was performed, using as probes either the skeletal muscle or erythrocyte acylphosphatase cDNA fragments described above. Skeletal muscle acylphosphatase mRNA accumulation peaked at 8 h (about 8-fold above control), while at 5 h no change could be observed. Further treatment with hormone resulted in a decrease in the mRNA level, down to two or three times control (Table 1). The same experiment performed using erythrocyte acylphosphatase cDNA as a probe showed that the mRNA specific to this isoform remained almost constant during the entire time course of the experiment (Table 1). As already shown in the experiment presented in Figure 2, the mRNA for this isoform was much more abundant than that for the muscle form.

Table 2 Effect of T_3-depleted medium on the subsequent accumulation of skeletal muscle acylphosphatase mRNA on stimulation with T_3

K562 cells were cultured in normal or T_3-depleted medium and then induced with 1 μg/ml T_3. Total RNA was subjected to Northern blot analysis, using the skeletal muscle acylphosphatase cDNA as a probe. Results were quantified by densitometric scanning with Cybertech CS-1 Image Analysis Software. Each value, expressed as a percentage of the control, was normalized to signals obtained using a human actin cDNA as probe on the same filters. The results are the means ± S.D. of three independent measurements.

	mRNA accumulation (%)	
Treatment period (h)	T_3-depleted FCS	FCS
0	100	100
8	785 ± 119	133 ± 23
24	232 ± 35	122 ± 22

The inducibility of several genes by thyroid hormones can be shown only with the use of depleted serum, probably because of receptor saturation. This was also true in the case of skeletal muscle acylphosphatase mRNA accumulation. A time course experiment was performed in normal or in depleted medium, after the addition of T_3. Under these conditions the mRNA levels were similar if the cells were cultivated in either normal or depleted medium, but inducibility by T_3 was virtually abolished in the presence of complete medium (Table 2). All the autoradiography data presented were normalized to an internal control; for this purpose the same filters were hybridized with a human actin probe, a gene that is not influenced by the addition of T_3.

In order to evaluate the minimal T_3 concentration capable of inducing acylphosphatase mRNA accumulation, a dose–response analysis was carried out. The skeletal muscle isoform mRNA level was measured 8 h after stimulation with different amounts of T_3, from 1.5 mM down to 10 nM. Results indicated that T_3 at a concentration of 180 nM is still active in inducing mRNA accumulation, although to a lower extent, while a concentration of 60 nM has no effect (results not shown).

Effect of T_3 on the transcription rate of the two isoforms of acylphosphatase

The accumulation of mRNA for skeletal muscle acylphosphatase during thyroid hormone treatment of K562 cells, shown by Northern blot analysis, could be due either to transcriptional regulation of gene expression or to post-transcriptional control of mRNA levels. In order to identify the mechanism involved in this phenomenon, we performed a nuclear run-on assay on isolated nuclei from K562 cells stimulated with T_3 for 4, 6 and 8 h. RNA polymerases engaged in transcription at the time of isolation of nuclei were allowed to elongate *in vitro* in the presence of [^{32}P]UTP. The ^{32}P-labelled RNAs were purified and hybridized to DNA probes for the two acylphosphatase isoenzymes, together with cDNAs of some genes not modulated by T_3, which were used as controls in the experiment; in this case we used human actin [18], chicken GAD [19] and human low-M_r PTPase [20]. The results are shown in Table 3. Treatment with T_3 clearly affected the synthesis of skeletal muscle acylphosphatase mRNA. At 4 h after the addition of hormone the transcriptional

Table 3 Effect of T_3 on the transcription of both skeletal muscle and erythrocyte acylphosphatases

Nuclear run-on assays were performed with K562 cells maintained in T_3-depleted medium and then treated with 1 μg/ml T_3. Cells were harvested after 4, 6 and 8 h and nuclei were isolated and treated as described in the Materials and methods section. A 2 μg sample of each DNA used as a template was bound to a Hybond N^+ filter and subjected to hybridization with the labelled transcripts. Autoradiography was for 3 days using a Phosphor Imager (Molecular Dynamics). Quantifications are expressed in arbitrary units and are the means ± S.D. of three independent measurements.

	Transcription (units)			
	Control	4 h	6 h	8 h
Human actin	4196 ± 279	3958 ± 196	4033 ± 245	4187 ± 205
PTPase	1496 ± 160	1383 ± 116	1301 ± 157	1215 ± 202
GAD	1011 ± 101	1248 ± 233	1246 ± 203	1116 ± 94.7
Skeletal muscle acylphosphatase	1816 ± 109	3505 ± 152	4444 ± 228	2200 ± 199
Erythrocyte acylphosphatase	6485 ± 269	6766 ± 245	6500 ± 288	6434 ± 295

Table 4 Effect of protein synthesis inhibitors on the accumulation of skeletal muscle acylphosphatase mRNA

K562 cells were cultivated in T_3-depleted medium; 24 h after seeding, cycloheximide or puromycin was added to the medium. After 2 h, 1 μg/ml T_3 was added and cells were harvested 8 and 24 h later. Total RNA was analysed by Northern blot using the skeletal muscle acylphosphatase cDNA as probe. Results were quantified by densitometric scanning with Cybertech CS-1 Image Analysis Software. Each value, expressed as a percentage of the control, was normalized to signals obtained using a human actin cDNA as a probe on the same filters. The results are the means ± S.D. of three independent measurements.

	mRNA accumulation (%)		
Incubation with T_3 (h)	No inhibitors	Cycloheximide	Puromycin
0	100	100	100
8	785.0 ± 119.0	112.7 ± 16.3	117.7 ± 23.1
24	232.0 ± 34.8	78.7 ± 15.8	312.7 ± 21.3

Table 5 Effect of T_3 on the accumulation of skeletal muscle acylphosphatase

K562 cells were treated for at least 8 days in medium containing T_3-depleted serum and then 1 μg/ml T_3 was added. ELISA determinations were carried out after 8 and 24 h, by a non-competitive sandwich test using serial dilutions of the lysate. The results are the means ± S.D. of three independent measurements.

Incubation with T_3 (h)	Skeletal muscle acylphosphatase (ng/mg of total protein)
0	12.5 ± 4.0
8	24.1 ± 6.0
24	25.7 ± 7.0

rate had doubled compared with the basal level, with a peak at 6 h. The basal rate was subsequently recovered after a further 2 h. These results are in agreement with the mRNA data reported above, indicating that the human muscle acylphosphatase gene is transcribed in the absence of T_3 stimulation, and that hormone treatment transiently increases the rate of transcription and subsequently the mRNA level.

In contrast, the transcription rate of the erythrocyte isoform did not appear to be modulated by T_3, as already suggested by the Northern blot data. It can be observed that the basal transcription rate was about three times higher than that of the muscle form.

Action of protein synthesis inhibitors

In order to investigate whether the T_3-dependent activation of the muscle acylphosphatase gene requires *de novo* protein synthesis, we used, in a Northern blot analysis, two well known inhibitors of translation: cycloheximide [24], which inhibits the peptidyltransferase step, and puromycin [25], which causes the premature termination of nascent peptides. K562 cells in culture were maintained in T_3-depleted serum for 8 days and then treated with 250 μM puromycin or 32 μM cycloheximide for 2 h before receiving hormone treatment. Total RNA was extracted 8 and 24 h after the addition of T_3 and hybridized with the skeletal muscle acylphosphatase cDNA probe. Results are shown in Table 4. The increase in the level of the muscle isoenzyme mRNA as a consequence of T_3 treatment was completely abolished by cycloheximide and appeared to be dramatically reduced by puromycin, while no significant effect was shown on the basal mRNA level. The amount of actin mRNA was unchanged even after 24 h, indicating a specific effect of the inhibitors on the acylphosphatase mRNA level. These results indicate that protein synthesis is necessary for T_3 induction of the acylphosphatase gene.

Effect of T_3 on skeletal muscle acylphosphatase content

We have shown above that T_3 can positively regulate skeletal muscle acylphosphatase mRNA content in K562 cells within a few hours. We also wanted to determine the kinetics and extent of accumulation of the enzyme in the cell in response to hormone treatment. For this reason an ELISA using anti-(muscle acylphosphatase) rabbit polyclonal antibodies was performed on hormone-treated cells. K562 cells (5×10^6) were cultured in T_3-depleted medium, and then induced with T_3 for 8 and 24 h. ELISA determinations of lysates were carried out by a non-competitive sandwich test using serial dilutions of the lysate. Table 5 reports the enzyme content of the cells at 8 and 24 h after addition of T_3. The increase in protein levels was very small (2-fold) in comparison with the increase in mRNA (6–10-fold) after 8 h of treatment with T_3; however, while the protein content continued to rise during the following 24 h, the mRNA content decreased rapidly almost to the steady-state level.

DISCUSSION

In this paper, we provide evidence that the expression of acylphosphatase is positively regulated by T_3: induction of

transcription by thyroid hormone may be one of the mechanisms for the regulation of expression of the skeletal muscle isoenzyme. In contrast, the other isoform of the enzyme, the erythrocyte acylphosphatase, is not influenced by T_3 administration. We have shown that treatment of K562 cells with T_3 causes a transient 10-fold increase in the accumulation of the mRNA coding for skeletal muscle acylphosphatase, but has no influence on the level of mRNA coding for the erythrocyte isoform (Table 1). Evaluation of transcriptional activity by nuclear run-on assays shows that transcription of the muscle acylphosphatase gene is transiently increased 2–3-fold over the basal level (Table 3). This observation could explain the transient accumulation of specific mRNA. Nevertheless, the possibility cannot be excluded that mRNA stability could play a role in the regulation of the mRNA level. Dual control of transcriptional regulation by T_3 has already been suggested for 'malic' enzyme [26,27].

It is generally accepted that stimulation of gene transcription by T_3 involves binding of the hormone to a nuclear receptor bound to *cis*-acting DNA elements within a gene's promoter or regulatory region [28]. The kinetics of the T_3-dependent stimulation of skeletal muscle acylphosphatase RNA transcription suggests a direct effect of T_3-interacting transcription factors on transcription initiation [29]: a small increase in acylphosphatase transcription is already detectable within 4 h and the maximal rate is achieved in about 6 h. It is conceivable, however, that additional factors are involved in transcriptional responses to hormones or other DNA-interacting agents. Thus the possibility cannot be excluded that T_3 may stimulate transcription of the skeletal muscle acylphosphatase gene via increased synthesis of one or more factors that regulate gene transcription.

The requirement for *de novo* protein synthesis is common among genes stimulated by T_3; both hydroxymethylglutaryl-CoA reductase and growth hormone genes show transcriptional regulation by T_3 that is dependent on ongoing protein synthesis [30,31]. Analysis of the effects of cycloheximide and puromycin on the accumulation of mRNA for skeletal muscle acylphosphatase (Table 4) reveals that, even though stimulation of gene transcription is rapid enough to be consistent with the direct involvement of a T_3 receptor, ongoing protein synthesis is absolutely necessary for the accumulation of cytoplasmic transcript. Our findings may indicate that the expression of the acylphosphatase gene is regulated either by a T_3-induced intermediate transcription factor or by the selective involvement of a short-lived protein co-operating in T_3-stimulated transcription. Either the latter possibility or a combination of both seems likely, because the relatively rapid response of skeletal muscle acylphosphatase to T_3 implies a direct involvement of the hormone in the activation of transcription.

The complexity of the regulation of the expression of skeletal muscle acylphosphatase is apparent if we consider the decrease in the accumulation of the specific mRNA for the enzyme at 24 h of stimulation with T_3, after reaching a maximum at 8 h. The effect on gene transcription is transient, probably due to a strong negative control. The same effect is observed with the nuclear transcript level, indicating that this negative control probably acts on transcription. It is in any case possible that mRNA stability could play an additional role in this phenomenon. The modulation of transcription of skeletal muscle acylphosphatase by thyroid hormone regulates the level of the enzyme in the cell, as shown by ELISA tests (Table 5); T_3 is capable of enhancing the concentration of the protein by a factor of two.

Our present findings agree with previous results on the effect of T_3 on acylphosphatase levels in animal tissues *in vivo* [9,11,32] and suggest that acylphosphatase may be partially involved in the overproduction of heat associated with hyperthyroidism. All known substrates of acylphosphatase are 'high-energy' phosphorylated compounds, so that this enzyme may be involved in energy metabolism pathways. Thus changes is acylphosphatase levels might provide a biochemical basis for the regulation of energy expenditure by means of futile cycles. For example, this enzyme causes an uncoupling of glycolytic ATP generation, since it catalyses the hydrolysis of 1,3-bisphosphoglycerate and causes an increase in the glycolytic rate [9,33,34]. Moreover, the 3-phosphoglycerate-dependent ATPase activity of acylphosphatase parallels the acylphosphatase-dependent increase in membrane Na^+/K^+- and Ca^{2+}-ATPase activities [4–6]. Both of these actions of acylphosphatase result in a loss of ATP-free energy and heat production; this mechanism may be responsible for thyroid hormone-dependent hyperthermia. Because of the correlation that can be established between hyperthyroidism and enhanced levels of acylphosphatase, K562 cells stimulated by T_3 may be a useful model for studying this kind of pathology.

In conclusion, the regulation of the expression of one of the acylphosphatase isoenzymes by thyroid hormone is via the control of transcription, although ongoing protein synthesis is required. Additional post-transcriptional mechanisms may also assist in making this effect transient. The lack of the same T_3-responsiveness for the erythrocyte acylphosphatase isoenzyme could be explained by the progressive loss of the *cis*-acting DNA elements that control T_3-induced transcription during the divergent evolution of the two genes coding for the two isoenzymes. Further work is needed to evaluate whether these differences accompany distinct physiological functions for the two acylphosphatase isoenzymes.

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