Cloning of a cDNA for the FAD-linked glycerol-3-phosphate dehydrogenase from rat liver and its regulation by thyroid hormones

(mitochondria/glycerol phosphate shuttle/quantitative PCR)

STEFAN MÜLLER AND HANS J. SEITZ

Institut für Physiologische Chemie, Abteilung für Biochemische Endokrinologie, Universitäts-Krankenhaus Eppendorf, 20246 Hamburg, Germany

Communicated by Henry A. Lardy, July 5, 1994

ABSTRACT A full-length 2.4-kb cDNA for the FAD-linked glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) was cloned from rat liver using PCR techniques. The cloned gene encodes a protein of 727 amino acids. The calculated molecular mass of 80,898 Da is higher than the apparent molecular mass observed by SDS/PAGE (74,000 Da) of the purified enzyme. This result indicates that the enzyme is synthesized as a precursor with a putative mitochondrial signal sequence. mRNA for this gene was detected in liver, heart, muscle, brain, testes, and pancreas. With the exception of testes, basal expression levels were very low in all tissues examined. However, application of thyroid hormones led to a 10- to 15-fold increase in liver glycerol-3-phosphate dehydrogenase mRNA, whereas hypothyroidism further decreased the mRNA level.

The FAD-linked mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH; EC 1.1.99.5) is a nuclear-encoded mitochondrial protein. It is located at the outer surface of the inner mitochondrial membrane (1) and catalyzes the conversion of glycerol 3-phosphate to dihydroxyacetone phosphate, thereby forming FADH₂ from FAD. This reaction is regarded as the rate-limiting step of the glycerophosphate shuttle (2). In this substrate shuttle, mGPDH works in conjunction with a NAD⁺-linked cytosolic glycerol-3-phosphate dehydrogenase (cGPDH; EC 1.1.1.8) to transfer cytosolic reducing equivalents, generated mainly during glycolysis, to the mitochondrial electron-transport chain. The enzyme is found in mammals (3–5), yeast (6), fungi (7), and bacteria (8).

Purified mGPDH from rat liver contains 1 mol of nonheme iron and 1 mol of noncovalently bound FAD per mol; it shows a molecular mass of ≈74,000 Da in SDS/PAGE (9) and is activated by Ca²⁺ (10). Whether the enzyme contains a cleavable presequence as a mitochondrial targeting signal or not is a matter of discussion (11, 12).

High levels of enzyme activity have been measured in insect flight muscle (13), in rat spermatozoa (14), and after thyroid hormone application in several rat tissues (15). The most dramatic thyroid hormone effect occurs in liver, where hyperthyroidism leads to a 20-fold increase in activity. In contrast, hypothyroidism results in a decrease to barely detectable levels of enzyme activity (15). Moreover, recent data (16) indicate an important role of mGPDH in glucose-sensing of the pancreatic beta cell, and the decrease of pancreatic mGPDH activity in animal models of type II diabetes (17) points to an involvement of this enzyme.

In this paper we report the nucleotide and deduced amino acid sequence of mGPDH, its expression in several rat tissues, and present our initial results on its regulation by thyroid hormones at the mRNA level.*

MATERIALS AND METHODS

Animals and Treatment. Adult male Wistar rats (Charles River Breeding Laboratories/Wiga Sutzfeld) were housed under controlled conditions (22°C, constant humidity, 12 hr/12 hr dark–light cycle); food and water were provided ad libitum.

Hypothyroidism was induced by i.p. injection of Na¹³¹I (Amersham) (250 μCi per 100 g of body weight; 1 Ci = 37 GBq) 28 days before the experiment. Hyperthyroidism was provoked either by a single i.p. injection of 3,5,3′-triiodo-L-thyronine (T₃) (20 μg per 100 g of body weight; Henning, Berlin) 48 hr before the experiment or by daily T₃ injections over 5 consecutive days with the last injection 24 hr before the experiment. Hypo- and hyperthyroidism were verified by measurement of serum T₃/thyroxine levels and of hepatic malic enzyme activity.

Enzyme Assays. Malic enzyme (18) and mGPDH (19) activity were determined as described.

Protein Purification. mGPDH was purified from rat liver according to Beleznaï and Jancsik (20). The final purification step was SDS/10% PAGE.

In Situ Proteolytic Cleavage and Peptide Microsequencing. Approximately 100 pmol of enzyme were separated on a SDS/10% PAGE, blotted to nitrocellulose (Schleicher & Schüll), and digested with either trypsin (Merck) or staphylococcus V-8 protease (Boehringer Mannheim) following strictly the protocol of Aebersold *et al.* (21). Fragments were separated by reversed-phase HPLC (Applied Biosystems Model 130 A) using a Vydac C₄ column. Not fully separated peaks were rechromatographed on a Vydac C₁₈ column. Microsequencing was done on a gas-phase sequenator (Applied Biosystems).

RNA Isolation. Total RNA was prepared as described (22). Poly(A)⁺ RNA was prepared using Dynabeads oligo(dT)₂₅ (Dynal, Oslo) according to the manufacturer's instructions.

Primer Design and cDNA Isolation by Reverse-Transcription (RT)-PCR. Forward primers for 3′ rapid amplification of cDNA ends (3′RACE) (23) were derived from trypsin peptide 1 and trypsin peptide 2. These oligonucleotides were designed according to the codon preference list described by Lathe (24). Deoxyinosine was incorporated at positions where a 4-fold degeneracy occurred, except toward the 3′ end. To avoid ambiguous positions within the five 3′ terminal bases two primers were synthesized for each peptide, differing only in one base (underlined below). Each primer consisted of 8-fold degeneracy. All were synthesized as sense

Abbreviations: mGPDH, FAD-linked mitochondrial glycerol-3-phosphate dehydrogenase; cGPDH, NAD⁺-linked cytosolic glycerol-3-phosphate dehydrogenase; T₃, 3,5,3′-triiodo-L-thyronine; RT-PCR, reverse-transcription PCR; RACE, rapid amplification of cDNA ends.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. X78593).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

primers, GP1a, 5'-GCIATCACIAATYTNGACGT; GP1b, 5'-GCIATCACIAATYTNGATGT; GP2a, 5'-GCITTTYTI-AATGTNCAGGC; GP2b, 5'-GCITTTYTIAATGTNC-AAGC.

First-strand cDNA was synthesized using the 3'RACE system (GIBCO/BRL). One microgram of poly(A)⁺ RNA was reverse-transcribed using the oligo(dT) adaptor primer [5'-GGCCACGCGTCGACTAGTAC(T)₁₇]. For cDNA amplification one of the degenerate primers (80 pmol) was combined with the adaptor primer (10 pmol) in a 50-μl reaction volume. The following PCR protocol was done in a Hybaid thermocycler (tube control mode): 95°C 4 min; 55°C 30 s, 72°C 90 s, 94°C 30 s, for 30 cycles; 55°C 30 s, 72°C 10 min. Hot start was done by adding 2.5 units of *Taq* polymerase (Pharmacia) after the initial denaturation step. Two rounds of amplification (nested PCR) (23) were done using all possible primer combinations. One microliter from the first-round PCR mixture served as template in the second round.

Ligation-anchored PCR was done as described (25). One microgram of poly(A)⁺ RNA was reverse-transcribed (Superscript; GIBCO/BRL) with the gene-specific primer GP4c, 5'-CAATCTTTGCCTCCTTGAAGG. A 5' phosphorylated

and 3' ddATP-blocked oligonucleotide anchor (5'-GATC-CAGGTCACCTTCAGGTCA) was ligated to the first-strand cDNA using T4 RNA ligase (Boehringer Mannheim). PCR conditions were as described above. The first round of PCR was done with the complementary anchor primer (5'-TGACCTGAAGTGACCTGGATC) and GP4c. The second round was done with the complementary anchor primer and the internal (nested) gene-specific primer GP5c (5'-CTTGTCTCTTCTGGAGCATGG).

Cloning and Sequencing. PCR products were recovered from low-melting-temperature agarose with Magic PCR Preps purification system (Promega) and cloned into pT7 Blue T-vector using the pT7 Blue T-vector kit (Novagen). Double-stranded sequencing was done by using the Sequenase 2.0 kit (United States Biochemical) with either universal sequencing primers or internal primers. For each fragment three clones from independent PCR reactions were sequenced in both directions.

Quantitative RT-PCR. The procedure described by Kandel *et al.* (26) was done. Five micrograms of total RNA from hypo-, eu-, and hyperthyroid liver was reverse-transcribed with 300 ng of random hexamer primers (Pharmacia) in a

GGTGAGGCGG	ACTGACTCCG	TTCTAGCTGG	AGGTTCTGGA	CTTCCCTGCT	CGCCGACCCC	TGGCGTGTGA	CGACCGATCT	CAGGCTGAGC	AATGGCGTTT	CAAAGGTAG	TGAAGGGAC	120
									MetAlaPhe	GlnLysValV	allYsglyTh	
TATTTCTATG	GGTGGAGGAG	CTCTGCCCAC	TGTTTTGGGA	CTCTCTCAGT	TTGCTCACTA	CAGAAGGAAG	CAAGTAAGCC	TGGCGTATGT	GGAAGCAGCA	ACATGCTTCT	CAGAGCCCGT	240
rileLeuMet	GlyGlyGlyA	laLeuAlaTh	rValLeuGly	LeuSerGlnP	heAlaHisTry	rArgArgLys	GlnValSerL	euAlaTyrVa	lgluAlaAla	ThrCysPheS	ergluProVa	
GAACAGGAG	CCTCCTTCCA	GAGAAGCTCA	GCTCATGACT	CTGCAGAAC	CATCGGAATT	TGACATCCTC	GTCATTGGAG	GAGGAGCCAC	AGGATGTGGC	TGTGGCGTAG	ACGCCGTCAC	360
laenArgGlu	ProProSerA	rgluValGlu	leuMetThr	leuGlnAsnT	hrSerGluPh	eAspIleLeu	ValIleGlyG	lyGlyAlaTh	rglyCysGly	CysAlaLeuA	spAlaValTh	
CAGAGGACTG	AAAACAGCCC	TTGTAGAGAG	AAATGACTTC	GCATCAGGGA	CTAGCAGCAG	AAGCACTAAA	TTGATCCATG	GTGGCGTACG	ATACCTCCAG	AAGGCTATCA	CGAACTTGGG	480
rArgGlyLeu	LysThrAlaL	euValGluAr	gaenAspPhe	AlaSerGlyT	hrSerSerAr	gSerThrLys	LeuIleHisG	lyGlyValAr	gTyrLeuGln	LysAlaIleT	hrAspLeuL	
TGTGGAACG	TATAGATGG	TGAAGAGGC	CCTTCATGAA	CGTGCCAAT	TACTAGAUA	CGCTCCCATC	CTATCAGCTC	CGCTGCCTAT	AATGCTGCCA	CTTTATAAGT	GTTGGCAGTT	600
pValGluGln	TyrArgMetV	alLysGluAl	leuHisGlu	ArgAlaAsnL	leuAlaGluT	eAlaProHis	LeuSerAlaP	roLeuProIl	LeuTyrLysT	rTrpGlnLe		
ACCTTATTAC	TGGGTGGGAA	TCAAGATGTA	TGATCTGGTT	CGAGGAAGTC	ATTGCGTGAA	GAGCAGTTAT	GTCTCAGTGA	AATCCAGAGC	CCTTGAACAT	TTCCCATGTC	TCCAGAGAGA	720
uProTyrTyr	TrpValGlyI	leLysMetTy	rasPheVal	AlaGlySerH	ieCysLeuY	sserSerTyr	ValLeuSerL	ysSerArgAl	aleuGluHis	PheProMetL	euGlnLysAs	
CAAGCTGGTC	GGAGCCATTG	TCTACTATGA	TGGACAGCAC	AATGACGCCC	GGATGAACCT	CGCCATCGCC	CTCACCCTGC	CCAGGTACGG	GCTGCCACG	GCCAATTACA	TGGAGTGGT	840
pLysLeuVal	GlyAlaIleV	alTyrTyrAs	pGlyGlnHis	AsnAspAlaA	rgMetAsnLe	uAlaIleAla	LeuThrAlaA	laArgTyrGl	ylaAlaThr	AlaAsnTyrM	etGluValVa	
GAGCTTGCTC	AAGAAGCAG	ACCCCGAACC	TGGGAAGAG	CGCGTGAGTG	GTGCGCGGTG	CAAGGACGTG	CTCAGAGGCG	ATGAGTTTAA	CCTGAGAGCT	AAATGCGTTA	TCAATGCCAC	960
lSerLeuLeu	LysLysThrA	spProGluTh	rgLysGlu	ArgValSerG	lyAlaArgCy	sLysAspVal	LeuThrGlyH	ieGluPheAs	nValArgAla	LysCysValI	leAsnAlaTh	
CGCCCTTTC	ACAGACTCCG	TGCGCAAAAT	GGATGATAAC	GATGTTGTTT	CCATCTGCCA	GCCAGTGCA	GGGGTTCACA	TTGTGATGCC	CGGGTACTAC	AGCCCGGAGA	ACATGGGACT	1080
rglyProPhe	ThrAspSerV	alArgLysMe	taAspAspAs	AspValValP	roIleCysGl	nProSerAla	GlyValHisI	leValMetPr	odglyTyrTyr	SerProGluA	snMetGlyLe	
TCTTGATCCT	GCAACAGCTG	ATGGCAGAGT	TATTTTCTTC	TTGCCCTTGG	AGAAGATGAC	AATTCGCGGT	ACCAGTGAAT	CGCCAACGGA	CCTCACGCGA	CATCTATTTC	CTTCGGAAGA	1200
uLeuAspPro	AlaThrSerA	spGlyArgVa	lilePhePhe	LeuProTrpG	luLysMetTh	rileAlaGly	ThrThrAspS	erProThrAs	pValThrHis	HisProIleP	roSerGluAs	
CGACATTAA	TTCACTTTAA	ATGAAGTGGC	GAACCTACCT	AGTTCGACG	TTGAAGTGAG	AAGAGGGAGT	GTCTCGGACG	CGTGGAAGTG	CATTCGCCCC	CTTGTACCG	ATCCCAAGTC	1320
pAspIleAsn	PheIleLeuA	snGluValAr	gaenTyrLeu	SerCysAspV	alGluValAr	gaArgGlyAsp	ValLeuAlaA	laTrpSerGl	yleArgPro	LeuValThrA	spProLysSe	
TGCAAACTCT	CAGTCCATCT	CTCGAAATCA	TGTGTGGGAA	GTGACGACAC	GTGGCCTCAT	CACAATAGCA	GTTGGGAAGT	GGACACATA	TGCTCCATG	GCAGAAGATA	CTGTGAACAA	1440
rAlaAsnThr	GlnSerIleS	erArgAsnHi	sValValGlu	ValSerAspS	erGlyLeuIl	eThrIleAla	GlyGlyLysT	rpThrThrTy	rArgSerMet	AlaGluAspT	hrValAsnLy	
AGCTGTCAAG	CTTCACAAC	TGAATGCAGG	ACCCAGTAGG	AACGTTGGGC	TCTTCTCTCA	AGGAGGCAAA	GATTTAGGCC	CCACACTCTA	CATCAGGCTT	GTCCAGGACT	ATGGGCTTGA	1560
sAlaValLys	LeuHisAsnL	euAsnAlaGl	yProSerArg	AsnValGlyL	euPheLeuGl	nglyGlyLys	AspTrpSerP	roThrLeuTy	rileArgLeu	ValGlnAspT	yrGlyLeuGl	
GAGTGAGGTT	GCACAACACT	TGGCCAAAAC	CTATGGTGAC	AAGCGTTTCG	ACGTGGCCAA	AATGGCAAGT	GTGACTGGAA	AACGGTGGCC	TGTTGTGGGA	GTGCGCTTTC	TGTGAGAATT	1680
uSerGluVal	AlaGlnHisL	euAlaLysTh	rTyrGlyAsp	LysAlaPheA	spValAlaLy	sMetAlaSer	ValThrGlyL	ysArgTrpPr	ovalValGly	ValArgLeuV	alSerGluPh	
TCCGTACTCT	GAAGCAGAGG	TGAATATATG	GATTAAAGAG	TACGCATGCA	CCGCTGTGTA	CATGATTTC	CGTCGCACCC	GCTTCGCTTT	TCTTAATGTT	CAGGCGACAG	AGGAAGCCCT	1800
eProTyrIle	GluAlaGluV	allLysTyrGl	yIleLysGlu	TyrAlaCysT	hrAlaValAs	pMetIleSer	ArgArgThrA	rgLeuAlaPh	eLeuAsnVal	glAlaAlaG	luGluAlaLe	
CCCAAGATT	GTTCAGTTAA	TGGGAAGAGA	ATTGAAGTGG	AGTGAATTGA	GGAAACAGGA	AGAAGTTGAA	ACAGCCACGA	GTTTCTGTGA	CTATGAAATG	GGCTATAAGT	CTCGAACAGA	1920
uProLysIle	ValGluLeuM	etGlyArgGl	leuAsnTrp	SerGluLeuA	rglyGlnGln	uGluLeuGlu	ThrAlaThrA	rgPheLeuTy	rTyrGluMet	GlyTyrLysS	erArgThrGl	
ACAACCTACA	GATAGTACTG	AAATCAGCCT	CGTGCCTCCA	GACATCGATC	GGTACAGAA	GAGATTTCAC	ATGTTTGAAT	AAGACGAAAA	AGGCTTCATT	ACCATTGTTG	ATGTTCAACG	2040
uGlnLeuThr	AspSerThrG	luIleSerLe	leuProPro	AspIleAspA	rgTyrLysLy	sArgPheHis	MetPheAsp	luAspGluLy	sglyPheIle	ThrIleValA	spValGlnAr	
TGTCTAGAG	AGTATCAATG	TACAAATGGA	CGAAGACACA	CTACATGAAA	TTCTCTGTGA	AGTAGACTTG	AACAAGAAAT	GTGAGTTGA	GCTCCATGAG	TTTCTGACAG	TGATGAGCGC	2160
gValLeuGlu	SerIleAsnV	alGlnMetAs	pGluAspThr	leuHisGluI	leLeuCysGl	uValAspLeu	AsnLysAsnG	lyGlnValGl	leuHisGluL	PheLeuGlnL	euMetSerAl	
AGTTCATACA	GGAAGGGTGT	CTGGAAGCCG	GCTTGCCATC	CTCATGAAGA	CTGCCGAGGA	GAAGTTGGAG	CGTAGAGTTC	CAATCCCGGT	GGATCGTAGT	TGTGGAGGAT	TGTGAGTCAL	2280
aValHisThr	GlyArgValS	erGlySerAr	gLeuAlaIle	LeuMetLysT	hrAlaGluGl	uAsnLeuAsp	ArgArgValP	roIleProVa	laAspArgSer	CysGlyGlyL	eu	
ACCAATAAAT	CCACAACCAA	CAAGCCTAGG	ACAGTCAGCA	CTATGTACAA	CCAGAGACGA	CTTAACACCA	TCTAAATAG	TGGCCATGTT	AGCTGCCTTT	TTTTTTTTTT	TTTTTTAA _n	2400

FIG. 1. Nucleotide and deduced amino acid sequence of rat liver mGPDH. Amino acid sequences derived from peptide sequencing are underlined. Primers used for cDNA isolation are boxed, and the direction of amplification is indicated by arrows. Note that primers GP1a,b and GP2a,b used in 3'RACE are derived from peptide sequences and, therefore, are degenerate. Primers for 5' end isolation of cDNA by ligation-anchored PCR (GP4c, GP5c) were synthesized as antisense primers. For PCR they were combined with a primer complementary to the 5' anchor.

20- μ l reaction volume. PCR analysis was performed by using primers for the internal standard (cGPDH) and for mGPDH. Primers for cGPDH were derived from the published mouse cDNA sequence (27). The forward primer (5'-CATTG-GCAAGATCTGTGACCT) matches positions 312–331; the reverse primer (5'-CTTCTCCAGCTGCTCAATGG) corresponds to nt 888–869. Primers for mGPDH amplification were as follows: forward, 5'-ATGGGACTTCTTGATCC-TGC, and reverse GP4c (see above). To compensate for the higher expression level of cGPDH and to assure that both target and control products can be analyzed within the exponential phase of the reaction, primers for cGPDH were added four cycles after PCR was started. Preliminary experiments showed an identical amplification efficiency for both products. Exponential kinetics were present up to 30 cycles. One tenth of the first-strand cDNA was used in a standard PCR reaction mixture containing 15 μ Ci of [α - 32 P]dCTP and 10 pmol of each primer. Samples (5 μ l) of the reaction were removed after 21, 23, 25, and 27 cycles and electrophoresed on a nondenaturing 4% polyacrylamide gel. Gels were dried and exposed to x-ray film. The bands corresponding to mGPDH and cGPDH were excised from the gel, and incorporated activity was measured by scintillation counting. Control reactions omitting the reverse transcription step were always done in parallel.

Tissue Distribution Studies. Northern blots were done as described (28). Five micrograms of poly(A)⁺ RNA was separated on a 1% agarose/formaldehyde gel, blotted onto Hybond-N (Amersham), and hybridized to a mGPDH cDNA probe. RT-PCR was done with random hexamer-primed (5 μ g of total RNA) first-strand cDNA from different tissues. 5'-CATGCTCCAGAAGGACAAGC served as forward primer, and GP4c served as reverse primer using the PCR conditions described above.

RESULTS AND DISCUSSION

Partial Amino Acid Sequence of mGPDH. Because the enzyme has been reported to contain a blocked N terminus (9), internal sequences after proteolytic cleavage of the purified enzyme with either trypsin or staphylococcus V-8 protease were obtained. The determined peptide sequences and their position within the protein are shown in Fig. 1.

cDNA Isolation. The most suitable peptide sequences for design of PCR primers were trypsin peptide 1 and 2. Two primers were derived from each peptide to reduce degeneracy and to create a stretch of five unambiguous bases at the 3' end of each primer (see *Materials and Methods*). In a nested 3'RACE PCR protocol one of these primers served as forward primer, and the oligo(dT) adaptor primer served as reverse primer. Nested 3'RACE PCR requires that the forward primer used in the second round of PCR is closer to the 3' end of the amplified sequence (internal) than the first-round forward primer. As the position of the peptides, and in consequence of the primers, was not known, all primer combinations had to be tested. By using primer GP1b in the first round and primer GP2a in the second round of PCR, an $\approx$ 600-bp PCR product was amplified (Fig. 1). Cloning and sequencing revealed this fragment to be part of the mGPDH cDNA because the deduced amino acid sequence contained V-8 peptides 2, 3, and 4 (Fig. 1). An additional $\approx$ 1300 bp of the cDNA were gained by PCR amplification using primer GP1b as forward and a reverse primer (GP3c) derived from the 5' region of the 600-bp fragment (Fig. 1). The remaining 5' end of the cDNA was isolated by 5' ligation-anchored PCR (see *Materials and Methods* and Fig. 1).

Nucleotide and Deduced Amino Acid Sequence. A cDNA of 2400 bp excluding the poly(A) tail was determined upon sequencing (Fig. 1). The cDNA sequence contains an open reading frame of 2181 nt coding for a protein of 727 amino acids with a calculated molecular mass of 80,898 Da. Two in-frame ATGs appear at positions 92–94 and 128–130, respectively. The first ATG (92–94) is assigned as the start codon because it matches the essential features for translation initiation described by Kozak (29). A putative polyadenylation signal is located at position 2284–2289, 107 nt upstream of the poly(A) tail. This result is atypical because usually the polyadenylation signal is found in the region from 10 to 30 nt upstream of polyadenylation. All peptide sequences could be identified in the deduced amino acid sequence.

From the deduced amino acid sequence one cannot infer the presence of a cleavable mitochondrial targeting sequence, as there is no N-terminal sequence available of the mature protein. However, the deduced N-terminal sequence (amino acids 1–40) is generally consistent with the features common

FIG. 2. Alignment of the deduced rat liver mGPDH amino acid sequence with yeast mGPDH (32) and aerobic GPDH from *B. subtilis* (33). Gaps introduced to maximize sequence homology are indicated by dashes. Amino acid residues identical in all three sequences are boxed. The putative FAD-binding domain (32, 33) is indicated and enclosed in a box (boldface type).

to mitochondrial targeting sequences (30)—i.e., enrichment of basic and hydroxylated amino acids and lack of acidic residues (Fig. 1). Furthermore, the presence of a signal sequence would explain the difference of ≈ 6 kDa between calculated and determined molecular mass. Using an *in vitro* translation approach Garrib and McMurray (11) detected a presumptive mGPDH precursor of ≈ 79 kDa. This molecular mass is very close to the calculated mass and supports the assumption of a 5- to 6-kDa leader sequence. In contradiction to that, others (12) could not show a mGPDH precursor by *in vitro* translation experiments. Therefore, the significance of the N-terminal sequence for targeting the protein to mitochondria has to be examined finally by functional studies.

As already observed for other sets of cytosolic and mitochondrial isoenzymes (31), the deduced protein sequence shares no homology with the cytosolic NAD⁺-linked GPDH from mouse (27). The alignment with FAD-linked GPDH from yeast (32) and *Bacillus subtilis* (33) shows an identity of 34 and 23%, respectively (Fig. 2). A highly conserved domain spans amino acids 71–122 (rat), where the three sequences are 63% identical. The putative FAD-binding site (32, 33) is located within this region (Fig. 2).

Tissue Distribution. In a Northern blot of RNA from different rat tissues a 2.4-kb RNA transcript could be detected in testes only (Fig. 3). In all other tissues examined the expression level was below the sensitivity of a conventional Northern blot. The relatively high expression level in testes correlates very well with the reported high enzyme activity in rat spermatozoa (14), which is consistent with the high rate of glycolysis and respiration in rat spermatozoa (34). By use of the RT-PCR technique with primers derived from the liver sequence, mRNA for mGPDH could be identified in liver, heart, kidney, muscle, brain, and pancreas (data not shown).

Regulation of mGPDH by Thyroid Hormones in Rat Liver. Because the enzyme activity of mGPDH has been reported to be dramatically increased by thyroid hormones (15), the question whether this regulation occurs at the mRNA level was addressed. As the basal level of mGPDH expression is very low, this question was approached with quantitative RT-PCR, a method shown to be thousands of times more sensitive than RNA blot techniques (35). As an internal standard cGPDH was coamplified with mGPDH because cGPDH mRNA levels were found to remain constant under various thyroid hormone conditions (data not shown). Thus, differences in gene expression of mGPDH in hypo-, eu-, and hyperthyroidism were measured by simply comparing the relative amounts of target (mGPDH) and standard (cGPDH) PCR product. The results of quantitative PCR are shown in Fig. 4. In hypothyroid animals mRNA levels of mGPDH were very low. Provoking hyperthyroidism in these animals by either a single T₃ injection 48 hr before the experiment or T₃ injection over 5 consecutive days led to a dramatic increase in mGPDH mRNA. Single application of T₃ resulted in a 10-fold induction, whereas increases after long-term appli-

FIG. 3. Detection of a 2.4-kb mRNA coding for mGPDH in rat testes. Autoradiogram of a Northern blot. Five micrograms of poly(A)⁺ RNA was separated on a 1% formaldehyde/agarose gel, blotted, and hybridized to a ³²P-labeled mGPDH cDNA probe.

FIG. 4. Effect of thyroid hormones on mGPDH mRNA in rat liver. A typical result of quantitative PCR is shown. RT-PCR was done as described. RNA from hypo- (lanes 1), eu- (lanes 2), and hyperthyroid (lanes 3) liver was used. Five microliters was removed from the PCR reaction at the times indicated and resolved on a 4% nondenaturing polyacrylamide gel. Dried gels were exposed to x-ray film for 1 hr. The upper band shows the 577-bp cGPDH fragment; the lower one shows the 346-bp mGPDH. Note that primers for cGPDH were added four cycles after the PCR was started. In the experiment shown here, hyperthyroid animals were T₃-treated for 5 days.

cation were up to 15-fold. Euthyroid animals exhibited only slightly higher (1.6-fold) mRNA levels than hypothyroid animals. Enzyme activity, measured in parallel, correlated very well with mRNA data (data not shown). This result strongly suggests that T₃ regulates mGPDH activity predominantly at the mRNA level and contrasts with a report (12) claiming that the availability of the FAD cofactor controls mGPDH activity under thyroid hormone influence. Further, the data from quantitative PCR indicates that in euthyroid liver expression of mGPDH is severalfold lower than expression of cGPDH.

The effect of thyroid hormones on mitochondrial oxygen consumption is a well-known phenomenon. However, little is known about the underlying mechanism(s). The thyroid hormone-mediated effect seems to be due to the coordinate transcriptional activation of proteins involved in, or linked to, the mitochondrial respiratory chain, such as cytochrome c (36), several cytochrome-c oxidase subunits (37), and the adenine nucleotide translocator (38). Our results show that mGPDH, which connects glycolysis with the mitochondrial respiratory chain, is also regulated at the mRNA level in rat liver. The up-regulation is paralleled by increased enzyme activity and may therefore contribute to the thyroid hormone-mediated increase in hepatic metabolic activity.

Note Added in Proof. Subsequent to the review of this manuscript, Brown *et al.* (39) reported the cloning of a cDNA for mGPDH from a rat testes library. Interestingly this sequence contains a different 5' untranslated region, whereas in the coding region it differs in 1 nt only (1482 C/A).

We thank Dr. F. Buck for separation and microsequencing of the peptides; K. Dümmler for providing RNA samples, helpful discussions, and advice; Dr. M. Harbers for help in protein purification; A. Harneit for excellent technical assistance; and Drs. T. Patschinsky and T. Pillar for discussing the manuscript. This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich-232.

- Klingenberg, M. (1970) *Eur. J. Biochem.* **13**, 247–252.
- Werner, H. V. & Berry, M. N. (1964) *Eur. J. Biochem.* **42**, 315–324.
- Hess, R. & Pearce, A. G. E. (1961) *Nature (London)* **191**, 718–719.
- Salganicoff, L. & Fukami, M. H. (1972) *Arch. Biochem. Biophys.* **153**, 726–735.
- Swierczynski, J., Scislowski, P. & Aleksandrowicz, Z. (1976) *Biochim. Biophys. Acta* **429**, 46–54.
- von Jagow, G. & Klingenberg, M. (1960) *Eur. J. Biochem.* **12**, 583–592.
- Courtright, J. B. (1975) *Arch. Biochem. Biophys.* **167**, 21–33.
- Kistler, N. S., Hirsch, C. A., Cozzarelli, N. R. & Lin, E. C. (1969) *J. Bacteriol.* **100**, 1133–1135.
- Garrib, A. & McMurray, W. C. (1986) *J. Biol. Chem.* **261**, 8042–8048.

10. Wernette, M. E., Ochs, R. S. & Lardy, H. A. (1981) *J. Biol. Chem.* **256**, 12767–12771.
11. Garrib, A. & McMurray, W. C. (1988) *J. Biol. Chem.* **263**, 19821–19826.
12. Clay, V. J. & Ragan, C. I. (1989) *Biochim. Biophys. Acta* **975**, 112–118.
13. Estabrook, R. W. & Sacktor, B. (1958) *J. Biol. Chem.* **233**, 1014–1019.
14. Schenkman, J. B., Richert, D. A. & Westerfeld, W. W. (1965) *Endocrinology* **76**, 1055–1061.
15. Lee, Y.-P. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1427–1436.
16. Sekine, N., Cirulli, V., Regazzi, R., Brown, L. J., Gine, E., Tamarit-Rodriguez, J., Girotti, M., Marie, S., MacDonald, M. J., Wollheim, C. B. & Rutter, G. A. (1994) *J. Biol. Chem.* **269**, 4895–4902.
17. Sener, A., Herberg, L. & Malaisse, W. J. (1993) *FEBS Lett.* **316**, 224–227.
18. Hsu, R. Y. & Lardy, H. A. (1969) *Methods Enzymol.* **13**, 230–235.
19. Garrib, A. & McMurray, W. C. (1984) *Anal. Biochem.* **139**, 319–321.
20. Beleznai, Z. & Jancsik, V. (1987) *Biochem. Int.* **15**, 55–63.
21. Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E. & Kent, S. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6970–6974.
22. Chirgwin, J. M., Przybyla, A. E., Mac Donald, R. J. & Rutter, W. J. (1977) *Biochemistry* **18**, 5294–5299.
23. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002.
24. Lathe, R. (1985) *J. Mol. Biol.* **183**, 1–12.
25. Troutt, A. B., McHeyzer-Williams, M. G., Pulendran, B. & Nossal, G. J. V. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9823–9825.
26. Kandel, J., Bossy-Wetzel, E., Radvanyi, F., Klagsbrun, M., Folkman, J. & Hanahan, D. (1991) *Cell* **66**, 1095–1104.
27. Dobson, D. E., Groves, D. L. & Spiegelman, B. M. (1987) *J. Biol. Chem.* **262**, 1804–1809.
28. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
29. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870.
30. von Heijne, G. (1986) *EMBO J.* **5**, 1335–1342.
31. Doonan, S., Barra, D. & Bossa, F. (1984) *Int. J. Biochem.* **12**, 1193–1199.
32. Rønnow, B. & Kielland-Brandt, M. C. (1993) *Yeast* **9**, 1121–1130.
33. Holmberg, C., Beijer, L., Rutberg, B. & Rutberg, L. (1990) *J. Gen. Microbiol.* **136**, 2367–2375.
34. Lardy, H. A., Ghosh, D. & Plaut, G. W. E. (1949) *Science* **109**, 365–372.
35. Wang, A. M., Doyle, M. V. & Mark, D. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9715–9721.
36. Scarpulla, R. C., Kilar, M. C. & Scarpulla, K. M. (1986) *J. Biol. Chem.* **261**, 4660–4662.
37. Wiesner, R. J., Kurowski, T. T. & Zak, R. (1992) *Mol. Endocrinol.* **6**, 1458–1467.
38. Luciakova, K. & Nelson, B. D. (1992) *Eur. J. Biochem.* **207**, 247–251.
39. Brown, L. J., MacDonald, M. J., Lehn, D. A. & Moran, S. M. (1994) *J. Biol. Chem.* **269**, 14363–14366.