Amino acid control of the human glyceraldehyde 3-phosphate dehydrogenase gene transcription in hepatocyte

Sophie Claeyssens, Christophe Gangneux, Carole Brasse-Lagnel, Philippe Ruminy, Toshihiko Aki, Alain Lavoinne, and Jean-Philippe Salier

MAMMALS CONSTANTLY ADJUST their metabolism in response to various physiopathological situations such as changes in nutritional status or other stressful conditions. The associated gene regulations are controlled by complex interactions of hormonal, neuronal, and nutritional factors. Unlike nutrients such as carbohydrates, lipids, vitamins, and minerals, whose roles in controlling gene expression have been extensively studied in mammals (23), the effect of amino acids (AA) has received limited attention (22, 32, 34, 37). An AA-regulated transcription has been reported for several genes, namely collagen 1α (10), β-actin (29), asparagine synthetase (AS) (25), and the C/EBP-homologous protein (CHOP) (18). However, only AS and CHOP transcription mechanisms have been elucidated at the promoter level, and quite a limited number of AA-responsive elements (AARE) has been described (9, 17, 27, 46, 47). Contrasting with the limited knowledge of the molecular mechanisms, whereby AAs control gene expression, the signaling pathways mediating the anabolic effects of some AAs on hepatic carbohydrate, lipid, and protein metabolisms (7, 8, 11) are being identified (22, 33, 50). These anabolic effects are similar to that of insulin in peripheral tissues, and experimental evidences demonstrate that AAs can interact with the insulin-signaling pathway. A recent study (33) indicates that the anabolic effects of Gln, Leu, and insulin are mediated by different signaling pathways and the latter two likely end up with shared targets.

The liver plays a central role in adaptation of metabolic pathways associated with the changes in AA metabolism and AA plasma content that are induced by nutritional factors or stress. Among relevant AAs, Gln plays a key role in stressful conditions where its liver uptake is markedly enhanced (49). On the other hand, glycolysis is one of the metabolic pathways most extensively involved in these situations, and the glycolytic enzyme GAPDH has a central role at this stage. Up to now, regulation of GAPDH gene expression has been poorly documented. Surgical stress and hepatocellular carcinoma have been shown to induce liver GAPDH mRNA expression (20, 26), whereas alcohol consumption decreased it (4). Regulation of GAPDH gene expression by nutritional and hormonal factors has also been reported. Notably, the hepatic expression of GAPDH is upregulated in fasted-refed rats (20, 38), but only stimulation by insulin has been documented...
(2). The insulin control of GAPDH is mediated by two independent insulin-responsive elements (IRE)-A and -B (38) that are located upstream of other regulatory elements involved in the basal activity of this gene (1). Furthermore, the GAPDH mRNA level is known to be controlled by a set of AAs (45), but a possible control of the GAPDH promoter by given AAs has not yet been documented. We have now investigated how insulin and AAs, mainly glutamine (Gln), regulate the hepatic expression of GAPDH. We demonstrate that insulin and Gln modulate the GAPDH promoter in an independent and gene-specific fashion and that Gln metabolism is required in the Gln-driven regulation. In the GAPDH promoter, an AARE that binds the C/EBPα and -δ transcription factors (TF) is responsible for this regulation by Gln.

MATERIALS AND METHODS

Cultures of HepG2 hepatoma cells. The human HepG2 hepatoma cell line was plated and maintained at 37°C under a 5% CO₂ and water-saturated atmosphere in an RPMI 1640 medium (Sigma) containing 2 mM L-Gln, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% (vol/vol) heat-decomplemented FCS. All induction experiments were carried out with subconfluent cells in an FCS-free medium. When indicated, a Gln-free RPMI 1640 medium was supplemented with Gln (Life Technologies) and/or insulin (Novo Nordisk), 6-diazo-5-oxo-l-norleucine (DON), glucosamine, or NH₄Cl (Sigma). In induction experiments with various AAs, an MEM medium (Life Technologies) lacking a given AA was supplemented or not with this AA at the concentration that is usually found in this medium, as indicated in RESULTS.

Determination of intracellular AA content. Subconfluent HepG2 cell cultures were washed with PBS and maintained for 24 h in the presence or absence of Gln. Then they were washed with PBS and scraped in 0.1 M sulfoosalicylic acid. The acid-precipitable material was discarded, and the AA content in the supernatant was measured on an amino acid autoanalyzer (Beckman).

RNA isolation and quantitation by Northern blot analysis. Total RNAs were extracted from HepG2 cells by an acidic guanidinium thiocyanate/phenol/chloroform procedure, electrophoresed (20 μg/lane), blotted, and hybridized with a [α-32P]dCTP-labeled cDNA as in Ref. 21. Full-length human cDNA probes included those for GAPDH (a kind gift from Dr. J. P. Borel, Centre National de la Recherche Scientifique (CNRS), Reims, France), α1-microglobulin/bikunin precursor (AMBP) (21), albumin (a kind gift from Dr. B. Bois-Joyeux, CNRS, Meudon, France), α2-HS glycoprotein (AHSG), and α1-antichymotrypsin (A1Ach; a courtesy from P. Arnaud, Charlestown, SC). A rat 18S cDNA was kindly provided by Dr. R. Vranckx (Paris, France). The autoradiographic signals obtained with a given cDNA probe were quantitated by densitometric scanning with an image analyzer (Biocom 500; Biocom, Les Ulis, France). In every experiment, we verified that the signal-to-amount ratio was linear in the range of mRNA amounts studied with each probe (not detailed). All densitometric values for a given mRNA were normalized with the 18S signals obtained on the same blot. Every value is the mean of at least three independent experiments with triplicates per experiment.

Plasmids. p47 Is a plasmid with the bacterial β-galactosidase (β-gal) gene under the control of the cytomegalovirus (CMV) immediate-early enhancer/promoter. pBLcat5 with the chloramphenicol acetyl-transferase (cat) gene driven by the Herpes simplex virus (HSV) thymidine kinase (tk) promoter (–109/+51) and the promoter-free pBLcat6 plasmid have been described elsewhere (14). The p488/cat plasmid with the human GAPDH promoter (–488/+21) fused to cat in pUCcat, the promoter-free pUC/cat plasmid, and deletion mutants of the GAPDH promoter covering from +21 to −329 (p329/cat), −308 (p308/cat), or −269 (p269/cat) in pUC/cat were a courtesy from Dr. X. Le Liepvre (41). p488/cat With the human GAPDH promoter (−488/+21) in pBLcat6 and its deletion mutants from +21 to −181 (p181/cat), −143 (p143/cat), or −81 (p81/cat) were a courtesy from Dr. T. Aki (1). Four mutants of p181/cat designated p181M1/cat to p181M4/cat, in which eight nucleotides in the natural GAPDH sequence were replaced by the 5′-TCTCGAGG-3′ sequence (an XhoI site is underscored) were purchased from BioMethods (Génopole Industries) and verified by sequencing. Other cat plasmids included p4964AMB/ cat with the human AMBP enhancer/promoter (from −4964 to +57) fused to cat in pUMSV0/cat and the control, promoter-free pUMSV0/cat plasmid (43) as well as p3147AHSG/cat with the human AHSG promoter (from −3147 to +48) fused to cat in pBL/cat6 (6).

HepG2 cell transfections with reporter plasmids and cat/β-gal assays. The HepG2 cells were plated in 6-cm plastic petri dishes and were first cultured in the standard plating medium described above. When the cells reached 70% confluency, the standard medium was replaced with MEM, and a cat plasmid (8 μg) and p47 (1 μg) were cotransfected by CaP04 precipitation (43). After 12 h, the cells were given media containing variable amounts of insulin and/or Gln (see RESULTS), further cultured for 24 h, and finally harvested by scraping. A cell extract was assayed for β-gal and cat activities, and the final values of cat activities were expressed as counts per minute (cpm)[3H]acetyl-chloramphenicol per β-gal unit (42). The background activity provided by a matched promoterless plasmid was subtracted from that obtained with the promoter/cat construct(s) under study. In every experiment, the transfection of each plasmid was done in triplicate. All final values provided in RESULTS are a mean calculated from at least three independent experiments.

EMSA. HPLC-purified oligos were purchased from Geneset. A series of double-stranded oligos covered the −127/−96 area in the GAPDH promoter, the wild-type (WT), and four mutant sequences noted (M1–M4), as further detailed in RESULTS. The WT sequence was used as a probe. Another probe was made of a proven C/EBP binding site (5′-CTAGGGCTTGGCAATCTATATTCG-3′, Geneka catalog ref. 1200009). Other double-stranded oligos harbored proven binding sites for CREB/ATF (5′-GATTCAGGATCATCACGCGCTGTG-3′, Geneka catalog ref. 1200007), upstream stimulatory factor (USF)-1 (5′-GGCCAGACACGGTGGCTCTGGTC-3′, Geneka catalog ref. 1200059), or NF-1 (5′-GGACACGTGGTTTACATTTGGACCAGCCACAGC-3′, Geneka catalog ref. 12011420). An AHSG double-stranded oligo, covering the −76/−50 area of the AHSG promoter, harbored a proven binding site for C/EBP (5). Probe labeling was done by end phosphorylation of one oligo with [γ-32P]ATP (5,000 Ci/mm; Amersham). Competitors were in a 200-fold molar excess over the GAPDH probe used at 1 ng/reaction or were in increasing molar excess over the C/EBP probe as indicated. Nuclear extracts from HepG2 cells were purchased from Eurocortex, and they were used at 10 μg/ml concentration. Electrophoresis was done in a nondenaturing 6% polyacrylamide gel (43). Rabbit polyclonal IgGs were purchased from Santa Cruz Biotechnology and included anti-CREB-1/ATF-1 (catalog ref. C-21), anti-CRE-BPI/ATF-2 (catalog ref. N-96), anti-ATF-3 (catalog ref. C-19), and anti-CRE-B2/ATF-4 (cat-
RESULTS

Effects of Gln and insulin on GAPDH mRNA level. We first evaluated whether Gln, alone or in association with insulin, can regulate the level of endogenous GAPDH mRNA in the human HepG2 hepatoma cells. As a control, we also investigated this effect on other genes with an hepatic expression, namely acute-phase proteins and genes A1Achy, albumin, AMBP, and AHSG (44). In HepG2 cells cultured for 48 h (Fig. 1), the GAPDH mRNA abundance increased with an increasing insulin input and plateaued at 20 mU/ml (resulting in a 3.3-fold increase), which is consistent with a previous report (38). The A1Achy, albumin, and AMBP mRNA levels peaked at 20 mU/ml. By contrast, the AHSG mRNA level remained unchanged. Whatever the amount of insulin, the GAPDH mRNA level increased (1.5–2 fold) in cells cultured with 10 mM Gln, whereas the A1Achy, albumin, AMBP, and AHSG mRNA levels were unaffected by this AA. A Gln-induced increase in GAPDH protein enzymic activity (1.4-fold) was consistent with these results (data not shown). Accordingly, Gln upregulates the GAPDH mRNA level in a gene-specific fashion and regardless of the insulin input.

Effects of Gln and insulin on GAPDH transcription. We next studied whether the above effects take place at a transcriptional level. Given that the endogenous expressions of the genes above are low in HepG2 cells, nuclear run-on experiments turned out to be poorly informative (data not shown). Therefore, we performed experiments with the GAPDH, AMBP, or AHSG promoters fused to the bacterial cat reporter gene. These promoter/cat constructs were transiently transfected in HepG2 cells. A culture period of at least 18 h was required to observe any insulin- or Gln-associated effect (data not shown). The transfected cells were cultured for 24 h in the absence or presence of 10 mM Gln and/or 20 mU/ml insulin. This insulin input was chosen because it induces a maximum upregulation of the GAPDH mRNA level (Fig. 1) and promoter activity (data not shown). As shown in Fig. 2, the GAPDH promoter activity was significantly increased in the presence of either insulin (2.5-fold), Gln (3-fold), or both (5.5-fold). These activities were additive, which is consistent with the insulin- or Gln-induced changes in endogenous GAPDH mRNA level observed above. Furthermore, the maximum and half-maximal Gln upregulation were respectively obtained with 2 and 0.5 mM Gln (Fig. 2). Therefore, the highest increase in Gln-induced transcription of GAPDH can be observed with the Gln content that is usually included in RPMI 1640 culture medium, namely 2 mM Gln. In contrast, the transcriptional activities of the AHSG and AMBP promoters were not affected by insulin, Gln, or both (Fig. 2). Likewise, a promoter of viral origin, namely the HSV tk promoter, was not affected. These features point to a GAPDH-specific transcriptional effect of insulin and Gln in our study.

Fig. 1. Influence of insulin and/or glutamine (Gln) on GAPDH, A1Achy, albumin, α1-microglobulin/bikunin precursor (AMBP), and α2-HS glycoprotein (AHSG) mRNA levels. HepG2 cells were maintained for 48 h in the presence of variable doses of insulin as indicated (abscissa) and in absence (open bars) or presence of 10 mM Gln (hatched bars). Total cellular RNAs were subjected to Northern blot analysis, and the same filters were successively probed with human GAPDH (A), A1Achy (B), albumin (C), AMBP (D), and AHSG (E) cDNAs. The signals obtained with a rat 18S cDNA were used for normalization between samples. Every mRNA level is expressed as %control level (100%) obtained in the absence of insulin and Gln. A significant difference (Student’s t-test for paired data; P < 0.05) between insulin vs. control ($) or between absence vs. presence of Gln at a given dose of insulin (*) is indicated.

AJP-Gastrointest Liver Physiol • VOL 285 • NOVEMBER 2003 • www.ajpgi.org
Specificity of the Gln-induced effect and associated metabolites. To determine the AA-dependent specificity of the Gln-induced effect, we compared the effects of various AAs. As shown in Fig. 3A, 0.1 mM Met upregulated the GAPDH promoter to a higher extent than Gln, whereas 0.05 mM Trp, 0.2 mM Phe, or 0.4 mM Val also upregulated this promoter, albeit to a low extent. Therefore, the Gln-induced effect is not strictly specific for this AA.

We next investigated whether the effect of Gln might be related to its transport system. Similar to Gln, Pro, His, and Asn are transported through an Na\(^+\)-dependent transport system in HepG2 cells (24). Yet, these amino acids do not modulate the GAPDH promoter (Fig. 3A). Even at a 10 mM concentration, Pro as well as Ala, which is also transported by an Na\(^+\)-dependent transport system (24), failed to upregulate the GAPDH promoter (data not shown). Conversely, Met and Trp, whose transport systems are Na\(^+\) independent, increased the GAPDH promoter activity (4- and 2-fold, respectively) when used at 10 mM (data not shown). This suggests that the Gln-induced effect is not related to its Na\(^+\)-dependent transport system.

We also investigated whether Gln metabolism is required in the process under study. Gln from the culture medium is metabolized by HepG2 cells, which, in turn, modulates the intracellular pool of free AAs. Indeed, we could observe that the total AA content of HepG2 cells increased 2.5-fold in the presence of 10 mM Gln (498 \pm 76 vs. 1,482 \pm 135 \mu mol/g protein). Whereas the contents of Gln and AAs resulting from Gln metabolism such as Glu, Ala, Asp, and Pro increased, Thr, Gly, Orn, and Asn contents were reduced and all other AA contents remained unchanged (data not shown). As shown in Fig. 3B, the inhibition of Gln metabolism by DON, an inhibitor of mitochondrial glutaminases and glutamine-dependent amidotransferases, abolished the effect of Gln on GAPDH transcription. Therefore, Gln metabolism is likely to be involved in the Gln-induced upregulation of GAPDH. Gln metabolism by glutaminase produces glutamate and NH\(_4\)Cl. A possible involvement of NH\(_4\)Cl was investigated. As shown in Fig. 3B, 10 mM NH\(_4\)Cl largely mimicked the Gln-induced upregulation of the GAPDH promoter. Finally, a potential role for glucosamine-6-phosphate, which is produced from Gln and fructose-6-phosphate by Gln fructose-6-phosphate amidotransferase, was also sought. Glucosamine mimicked the Gln-induced effect as its input in the culture medium strongly increased the GAPDH promoter activity. These results indicate that Gln metabolism is a neces-
decreased it (data not shown), which is consistent with each tested effect relative to the Gln effect set as 1. This is because the promoter activity, whereas deletions down to Gln added or not with 6-diazo-5-oxo-L-norleucine (DON; 4 mM) as cells cultured for 24 h in the absence (−) or presence (+) of 10 mM DON. The transcriptional activity of the GAPDH construct p488/cat was tested in HepG2 cells cultured for 24 h in the absence of Gln are also shown. Every effect is calculated as a percentage of p488/cat activity obtained in absence of Gln, and the bar represents the Gln effect set as 1. The effects of glucosamine (10 mM) or NH₄Cl (10 mM) in the absence of Gln were tested in HepG2 cells cultured for 24 h in the absence (-) or presence (+) of Gln metabolism vs. Gln. The transcriptional activity of the GAPDH promoter between bp −143 and −83, which acts independently of IRE-A, IRE-B, and other upstream elements previously described.

As shown in Fig. 4 (bottom left), the −116/−105 sequence in GAPDH exhibits some similarity with the AARE at −70/−64 and the nutrient-sensing response element 1 (NSRE-1) at −68/−60 found in the AS promoter (9, 27). Also, a search with the program MatInspector combined with the TRANSFAC database software (52) revealed that the GAPDH sequence at −126/−118 exhibits a similarity with binding sites for the ATF/CREB, C/EBP, and USF families and the sequence at −110/−97 is somewhat similar to a binding site for the ATF/CREB family. To assess the possible involvement of this −127/−96 sequence in the response to Gln, a series of p181M1/cat to p181M4/cat were used to scan this sequence and were tested for cat activity (Fig. 5). The mutations M1 and M2 slightly decreased the basal activity of the GAPDH promoter, whereas the mutation M4 slightly increased it. Moreover, M2, M3, and M4 did not prevent the Gln-induced upregulation of the GAPDH promoter, whereas M1 suppressed it. Therefore, the −116/−105 sequence that is similar with the AARE and NSRE-1 in AS does not participate in this activation. By contrast, the −126/−118 sequence that is similar to ATF/CREB, C/EBP, and USF binding sites mediates the Gln-induced upregulation of the GAPDH promoter. This element is here below designated as the GAPDH Gln-responsive element (GlnRE).

Transcription factors involved in GAPDH activation by Gln. EMSAs were performed with a GAPDH probe covering from −127 to −96 (Fig. 6). Four bands formed with HepG2 nuclear extracts (lane 1). These bands included two specific complexes, A and B, as proven by an autologous competitor (lane 2), as well as two non-specific complexes (*). Complex A was detected as a slow, faint band, whereas complex B appeared as a strong band of fast mobility. To identify which GAPDH sequence(s) was able to form complex A or B, mutant oligos M1–M4, whose sequences were identical to those shown in Fig. 5, were used as competitors. Complex A was abolished by M2, M3, and M4 (lanes 4–6) but not by M1 (lane 3), whereas complex B was abolished by M1 and M2 (lanes 3 and 4) but by neither M3 nor M4 (lanes 5 and 6). The locations of the mutated nucleotides in M1–M4 indicate that two sequences at −127/−120 and −110/−97 participate in the formation of complexes A and B, respectively. Because these sequences encompass the potential ATF/CREB-, C/EBP-, or USF-binding sites noted above, proven binding sites...
for some members of these families were used as competitors. A binding site for ATF/CREB (lane 7) or C/EBP (lane 9) abolished complex A, whereas an USF-1 binding site failed to do so (lane 8), indicating that complex A at −126/−118 is C/EBP and/or ATF/CREB related. Also, the ATF/CREB competitor abolished complex B (lane 7), indicating that complex B at −110/−97 is ATF/CREB related. A competitor with an NF-1 site used as a negative control failed to abolish either complex A or complex B.

Supershifts (Fig. 6, lanes 11–19) were performed to identify which members of the C/EBP and ATF/CREB families form complexes A and B. Anti-C/EBP-α antibodies abolished complex A (lane 12), and anti-C/EBP-β
antibodies supershifted it (lane 14), whereas anti-C/EBP-β (lane 13), -CREB-1/ATF-1 (lane 16), -CREB-2/ATF-4 (lane 17), -ATF-3 (lane 18), or -USF-1 antibodies (lane 19) did not. Therefore, complex A is made up of C/EBP-α and -δ proteins. Anti-CRE-BP1/ATF-2 antibodies supershifted complex B (lane 15), whereas anti-C/EBP-α (lane 12), -β (lane 13), -δ (lane 14), -CREB-1/ATF-1 (lane 16), -CREB-2/ATF-4 (lane 17), -ATF-3 (lane 18), or -USF antibodies (lane 19) did not. Therefore, complex B is made up of the CRE-BP1/ATF-2 protein. Of note, the supershifted complex B was relocated over the EMSA area normally occupied by complex A. Hence, it cannot be formally excluded that the anti-CRE-BP1/ATF-2 antibodies supershifted complex A as well. Finally, together, our mutagenesis experiments and EMSA data pointed to the binding site at −127/−96 and the associated complex A as the mediators of the GAPDH promoter regulation by Gln. To confirm that the binding site at −127/−96 is a bona fide C/EBP binding site, a reverse EMSA experiment with a proven C/EBP binding site used as a probe was carried out (Fig. 6, lanes 20–32). The complexes formed with this probe and an HepG2 nuclear extract included a nonspecific complex (c) as well as a major set of closed bands (noted C) whose appearance fits the pattern expected for C/EBP complexes (3). This set of bands was competed out by the autologous competitor (lanes 21–23), a heterologous, high-affinity C/EBP binding site found in the AHSG promoter (5); and data not shown) (lanes 30–32) and the WT GAPDH sequence −127/−96 (lanes 24–26) but was not competed by the GAPDH mutant M1 (lanes 27–29). This unambiguously identified the −127/−120 sequence as a C/EBP binding site. Moreover, the above competitions that were done with a variable excess of competitor pointed to a similar affinity of the C/EBP proteins for the site at −127/−120 in GAPDH and the high affinity site in AHSG, thus indicating that the former site has a strong affinity for C/EBP proteins.

DISCUSSION

Our data document the novel observation that Gln is able to upregulate GAPDH gene expression in an additive way with insulin. This event takes place, at least partly, at the transcriptional level and is consistent with an increase in in vivo transcription rate in rat hepatocytes incubated with Gln as observed in run-on experiments (data not shown). This effect is gene specific, because Gln is unable to modulate the hepatic
transcription of some other genes, namely AMBP and AHSG. Studies of the AA-controlled expression of the AS and CHOP genes have shown that they specifically and differently respond to availability of various sets of AAs (25, 31). Consistent with this, the overall pattern of GAPDH gene expression in response to given AAs is different from what is observed for CHOP and AS genes. A noted exception is Met, which is a potent inducer for AS, CHOP, and GAPDH. Moreover, GAPDH expression is controlled by a set of essential AAs (i.e., Met, Phe, Trp, and Val) and by Gln, considered a "conditionally essential" AA (12). This similar effect of essential AAs and Gln underscores the Gln characteristic. Moreover, these AAs effects occur at physiological concentrations as observed in portal vein of fed rats (30). Likewise, half-maximal Gln activation of GAPDH transcription occurs at 0.5 mM, the physiological serum Gln concentration in human.

Gln exerts anabolic effects on liver metabolism by stimulating glycolysis, lipid, and protein metabolisms, and most of these Gln-induced effects are mediated by cell swelling that results from a Na⁺-dependent transport of AAs, a subsequent accumulation of catabolic byproducts, and an associated increase in intracellular osmolarity (11, 33, 50). These Gln effects were reproduced in our experimental conditions. Indeed, in HepG2 hepatoma cell cultures, Gln is transported through an Na⁺-dependent transport system (24), and a Gln/glutaminase-mediated production of Glu as well as a production of Asp and Ala by Glu transamination most likely contributed to the observed accumulation of these AAs. Yet, Pro and Gly, whose intake is Na⁺ dependent (24), failed to regulate GAPDH transcription. Moreover, Met and Trp, which are usually transported through a Na⁺-independent system in mammalian cells, upregulated GAPDH transcription. Together, these features point to a cell-swelling independent mechanism in the Gln-mediated GAPDH regulation.

Gln metabolism is important in regulating GAPDH gene transcription. Indeed, 1) the Gln-induced upregulation of the GAPDH promoter was time (>18 h) and dose dependent, 2) an inhibition of Gln metabolism suppressed GAPDH activation, 3) Gln byproducts such as ammonia, which is produced by glutaminase, and glucosamine, which is produced by Gln fructose-6-phosphate amidotransferase, also activated GAPDH transcription. Glucosamine, which is likely phosphorylated into glucosamine-6-phosphate in HepG2 cells, is the precursor of the hexosamine pathway. Interestingly, this pathway leads to protein glycosylation and is known to participate in signal transduction (51).

We have mapped the GAPDH GlnRE to a GCACG-/TAGC motif at −126/−118. This excludes that the two upstream elements involved in the basal GAPDH expression (1) as well as the IRE-A and -B sites participate in the Gln responsiveness of this gene. Our supershift experiments have indicated that the GAPDH GlnRE binds C/EBPα and -δ. The identification of these TFs is compatible with a previous detection of a C/EBP binding site around −100 (2). All members of the C/EBP family contain a bZIP domain. The latter consists of a basic AA-rich DNA binding region and a leucine zipper dimerization motif that mediates a homo- or heterodimerization with other C/EBP proteins (40). Such a heterodimerization between C/EBPα and -δ over the GAPDH GlnRE is suggested by the complete removal of complex A by both anti-C/EBPα and -δ antibodies. Also, the C/EBP proteins can heterodimerize with other bZIP TFs such as the CREB/ATF family members (28, 40). In keeping with this, a competitor oligo with a CREB/ATF site abolished the formation of complex A in our EMSAs. Therefore, the further participation of a CREB/ATF protein in some C/EBP heterodimers bound to the GAPDH GlnRE is to be considered. Indeed, 1) the pattern of complex A over the GAPDH probe is different from that of typical complex C over a control C/EBP probe, and 2) mobility of complex A is slower than that of complex C in keeping with ATF2 size (55 kDa), which is almost twice that of the main isoforms of C/EBPα and -δ. Our finding that the Gln-driven transcription of GAPDH is mediated by C/EBP is consistent with the acknowledged role of these TFs in controlling the expression of genes that play a central role in the carbohydrate and lipid metabolisms (35, 40). C/EBP expression is regulated by numerous pathways including those for some hormones and nutrients (40). In particular, an AA limitation (Phe, Met, Leu, or Trp) increases the abundance of C/EBPα and -β mRNAs in rat hepatoma cells (37). However, neither the migration nor abundance of the (GlnRE-C/EBP) complex A seen in our EMSA changed following Gln induction (data not shown). Therefore, our data suggest that the Gln-induced up-regulation of GAPDH transcription does not involve any change in C/EBP binding activity. Because GAPDH activation requires Gln metabolism and is also induced by glucosamine, the possibility that Gln controls GAPDH transcription by virtue of a change in TF glycosylation is an attractive hypothesis that deserves further investigation.

In the GAPDH promoter, the sequence starting at 8 nt downstream of the GlnRE is somewhat similar to AS or, to a lesser extent, CHOP AAREs. However, this AS sequence that binds ATF-2 does not mediate the Gln responsiveness. Yet AS or CHOP AAREs are, in turn, only partially structurally and functionally related (16). Indeed, The AS AARE is made up of two responsive elements (NSRE-1 and -2) that are involved in response to AA starvation or an endoplasmic reticulum stress (notably induced by glucose starvation). C/EBPβ(h8) and ATF-4(h7) bind to NSRE-1, are the mediators of these responses, and are transcriptionally regulated by nutrients. On the other hand, ATF-2, which does not bind to NSRE-1, is necessary to obtain the full AS response to the above stress. The CHOP AARE shares homology with the AS NSRE-1, but it binds ATF-2 and responds to AA starvation only. Moreover, the AS promoter harbors three GC-rich sequences that are located about 30 nt upstream of the AARE and are required for the AA-induced transcription of this gene (36). The GAPDH promoter also con-
tains two GC-rich sequence boxes that are located 30 nt upstream of the GlnRE (−181/−143) and allow for a high basal transcription rate (1). However, these GC-rich sequence boxes are not required for an induction of GAPDH by AAs. Collectively, our data show that the GAPDH GlnRE is structurally and functionally different from AS and CHOP AAREs.

Although extrapolation of our results to human liver must be made with caution in view of the limitations inherent to the use of a cell line as an experiment model, the hepatic regulation of GAPDH by Gln and some essential AAs might be of physiological relevance. The AA-induced GAPDH upregulation might be associated with an increased glycolytic flux yielding the substrates and energy required for some anabolic processes. This hypothesis is consistent with the observation that parenteral Gln infusion enhances glucose use in dog (13). Alternatively, an AA-induced GAPDH upregulation might also be important for the potential nonglycolytic functions of the enzyme. Indeed, over the last decade, numerous studies have identified diverse biological properties for mammalian GAPDH protein. GAPDH has a role in membrane fusion and transport; in the cytoplasm, it participates in the translational control of gene expression by phosphorylation as well as in microtubule assembly; within the nucleus, it is involved in tRNA export and DNA replication and repair. It also may play a role in apoptosis, neuronal disorders, and prostate cancer, and it appears to be involved in the toxicology of nitric oxide (46). A better knowledge of AA effects at a molecular level will likely help optimize the use of AAs as a nutritional support well beyond some current conventional treatments. In particular, it is noteworthy that the GAPDH response to Gln is insulin independent. Yet some AA-associated beneficial effects on carbohydrate and protein metabolism are emerging from clinical investigations. Indeed, an oral supplementation with essential AAs for patients with Type 2 diabetes mellitus appears to decrease postprandial plasma glucose (39). Likewise, enteral Gln stimulates gut protein anabolism as reflected by an increased mucosal fractional synthesis rate in humans (15, 19). Identification of novel target genes for AA-based adjuvant therapies remains an important challenge for the future.

We are grateful to Dr. M. C. Alexander-Bridges who made possible the use of p488/cat plasmid.

REFERENCES


