Transcriptional regulation of the human asparagine synthetase gene by carbohydrate availability

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Transcription of the asparagine synthetase (*AS*) gene is induced by amino acid deprivation. The present data illustrate that this gene is also under transcriptional control by carbohydrate availability. Incubation of human HepG2 hepatoma cells in glucose-free medium resulted in an increased AS mRNA content, reaching a maximum of about 14-fold over control cells after approx. 12 h. Extracellular glucose caused the repression of the content of AS mRNA in a concentration-dependent manner, with a $k_{1/2}$ (concentration causing a half-maximal repression) of 1 mM. Fructose, galactose, mannose, 2-deoxyglucose and xylitol were found to maintain the mRNA content of both AS and the glucose-regulated protein GRP78 in a state of repression, whereas 3-*O*-methylglucose did not. Incubation in either histidine-free or glucose-free medium also resulted in adaptive regulation of the *AS* gene in BNL-CL.2 mouse hepatocytes, rat C6 glioma cells and human MOLT4 lymphocytes, in addition to HepG2 cells. In contrast, the steady-state mRNA content of GRP78 was unaffected by amino acid availability. Transient transfection assays using a reporter gene construct documented that glucose deprivation increases *AS* gene transcription via elements within the proximal 3 kbp of the AS promoter. These results illustrate that human *AS* gene transcription is induced following glucose limitation of the cells.

Key words: amino acids, carbohydrate response element, glucose response element, metabolite control, nutrient control.

INTRODUCTION

Both major and minor nutrients participate in the regulation of gene expression in response to nutritional changes in mammals (reviewed in [1–4]). Changes in cellular carbohydrate levels cause the transcriptional regulation of a number of genes that encode proteins associated with lipid and carbohydrate transport/metabolism. The presence of carbohydrate causes an increased transcription of the genes for L-type pyruvate kinase, fatty acid synthase, acetyl-CoA carboxylase, S_{14} protein [3,4] and the glucose transporter GLUT2 [5]. In contrast, depriving cells of glucose results in increased transcription of the genes for the glucose-regulated proteins GRP78 and GRP98 [6], as well as the glucose transporters GLUT1 [7,8] and GLUT3 [9].

Most mammalian cells express the enzyme asparagine synthetase (AS; EC 6.3.5.4), which catalyses the biosynthesis of asparagine from aspartate and glutamine while hydrolysing ATP to AMP and pyrophosphate. The *AS* gene/cDNA has been cloned from a number of organisms [10]. The amino-acid-dependent regulation of *AS* has been well documented. In 1977, Arfin et al. [11] showed that starvation of Chinese hamster ovary (CHO) cells of asparagine decreased the level of charged $tRNA^{Asn}$ molecules and, subsequently, AS enzymic activity was increased. More recently, Basilico and co-workers identified AS as being the protein able to complement a temperature-sensitive baby-hamster-kidney-cell-cycle mutant [12,13]. While investigating the mechanism of the cell-cycle block, the authors also determined that the level of AS mRNA increased in cells deprived of asparagine, leucine, isoleucine or glutamine [14]. Hutson and Kilberg [15] cloned a rat AS cDNA and demonstrated an increased AS mRNA content in response to amino acid deprivation in rat Fao hepatoma cells in culture [15]. Depletion of a single essential amino acid, such as histidine, from an otherwise complete culture medium, or inhibition of histidinyl-tRNA synthetase by the amino acid analogue histidinol, caused an elevation of the steady-state AS mRNA level.

During carbohydrate deprivation in mammals, protein and lipid catabolism takes place to supply energy for other cellular functions. In addition to amino-acid-dependent regulation, and analogous with the sugar-dependent regulation of asparagine synthesis in plants [17,18], we hypothesized that the mammalian *AS* gene might also be transcriptionally controlled by sugar availability. In the present study, we have studied the changes in the expression of the human *AS* and *GRP78* genes under conditions of glucose starvation in HepG2 cultured hepatoma cells. Both AS and GRP78 mRNA accumulated during glucose starvation. However, in contrast with the increase in AS mRNA, the GRP78 mRNA content was unchanged in response to amino acid deprivation in HepG2 cells. We also demonstrated that the accumulation of AS mRNA can be partly prevented by the presence of 2-deoxy-D-glucose (DOG), and completely blocked by fructose, galactose, mannose and xylitol, but not 3-*O*-methylglucose (3OMGlc), suggesting that glucose metabolites are required for the maintenance of the *AS* gene in its repressed state. Glucose-dependent expression of a growth hormone (GH) reporter gene was observed when under the control of a 3-kbp human *AS* gene-promoter fragment, demonstrating the transcriptional basis of the increase in steady-state AS mRNA in carbohydrate-starved HepG2 cells.

EXPERIMENTAL

Cell culture

The following cell lines were obtained from American Type Culture Collection: human hepatoma HepG2, human lymphoma

Abbreviations used: AS, asparagine synthetase; CHO, Chinese hamster ovary; ChoRE, carbohydrate response element; DEPC, diethyl pyrocarbonate; DOG, 2-deoxy-D-glucose; FBS, fetal bovine serum; GH, growth hormone; MEM, minimal essential medium; 3OMGlc, 3-*O*-methylglucose.

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MOLT4, mouse fetal liver BNL-CL2 and rat glioma C6. Cells were cultured in minimal essential medium (MEM) [19], pH 7.4, supplemented with 25 mM $NaHCO_3$, 2 mM glutamine, 10 μg/ml streptomycin sulphate, 100 μg/ml penicillin G, 28.4 μg/ml gentamycin, 0.23 μg/ml *N*-butyl-*p*-hydroxybenzoate, 0.2 % (w/v) BSA and 10 % (v/v) fetal bovine serum (FBS). Cells were maintained at 37 °C in a CO_2/air incubator (1:19) in T-75 flasks, and those flasks containing the MOLT4 cells in suspension were maintained on a rocking platform. To induce the expression of the *AS* gene, cells were transferred to either 60- or 100-mm dishes (for attached cells), or a T-75 flask (MOLT4 cells only), cultured to near-80 % confluence, and then incubated in complete MEM, glucose-free MEM or histidine-free MEM, pH 7.4, supplemented with 25 mM $NaHCO_3$, 2 mM glutamine, 10 μg/ml streptomycin sulphate, 100 μg/ml penicillin G, 28.4 μg/ml gentamycin, 0.23 μg/ml *N*-butyl-*p*-hydroxybenzoate and 10 % (v/v) dialysed FBS.

Immunoblotting

Tissue culture dishes (100 mm) were rinsed with PBS, 1 ml of sample dilution buffer [124 mM Tris/HCl (pH 6.6)/1 % (w/v) SDS/700 mM 2-mercaptoethanol/20 % (v/v) glycerol/30 μg/ml Bromophenol Blue] was added, and the dish was then incubated on ice for 15 min. After incubation, the protein homogenates were scraped, transferred to a microcentrifuge tube and centrifuged at 10000 ***g***. Equal amounts of supernatant protein were subjected to SDS/PAGE (7.5 % gels), and immunoblotted using a hybridoma medium containing a monoclonal antibody raised against AS, as described previously [16]. The secondary antibody was goat anti-(mouse IgG) linked to horseradish peroxidase (Gibco BRL, Gaithersberg, MD, U.S.A.), and this was detected using an enhanced chemiluminescence system, according to the manufacturer's instructions (Amersham, Arlington Heights, IL, U.S.A.).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated by the method of Chomczynski and Sacchi [20], but with a few modifications. Briefly, the monolayer cells, cultured as described above, were washed once with PBS [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)], and then 2 ml/60-mm dish or 3 ml/100-mm dish of a guanidinium thiocyanate solution [4 M guanidinium thiocyanate/25 mM sodium citrate (pH 7.0)/0.5 % (w/v) sarcosyl/0.1 M 2-mercaptoethanol] was added. The MOLT4 cells were collected by centrifugation at 300 ***g*** for 5 min, washed once with PBS, and then 3 ml of the guanidinium thiocyanate solution was added to the pellet. The homogenate was collected in a 15 ml conical polypropylene tube and extracted with 0.1 vol. of 2 M sodium acetate, pH 4.0, and an equal volume of water-saturated phenol. This suspension was mixed together and cooled on ice for at least 15 min. Before centrifugation at 3000 ***g*** for 20 min at 4 °C, 0.22 vol. of chloroform/3-methylbutan-1-ol (49:1) were added, and the mixture was shaken vigorously for 20 s. After centrifugation, the aqueous phase was transferred to a fresh centrifugation tube and mixed with an equal volume of propan-2-ol. This solution was placed at −20 °C for at least 1 h and then centrifuged at 10000 ***g*** for 25 min at 4 °C. The RNA pellet was dissolved in 500 μl of guanidinium thiocyanate solution and transferred to a 1.5-ml Microfuge tube. An equal volume of propan-2-ol was added and the mixture was incubated for at least 1 h at −20 °C. RNA was collected by centrifugation at 10000 ***g*** for 10 min at 4 °C, and 400 μl of diethyl pyrocarbonate (DEPC)-treated water was added to the pellet that was incubated at 50 °C until completely resuspended. The RNA was precipitated with 0.1 vol. of DEPC-treated 3 M sodium acetate, pH 5.2, and 2.2 vols of 100 % ethanol, followed by incubation at −20 °C for at least 1 h. The RNA was collected once more by centrifugation, resuspended in 300 μl of DEPC-treated water and ethanol-precipitated once again. The final pellet was dried to remove traces of ethanol, dissolved in DEPC-treated water, and the RNA concentration was estimated by measuring A_{260}.

Total RNA (20 μg) was dried and resuspended in 25 μl of 12.5 M formamide/6.6 % (v/v) formaldehyde/6 mM sodium acetate, pH 7.4/0.5 mM EDTA, pH 8.0/20 mM Mops, pH 7.0. This RNA was dissolved and denatured by subsequent incubations for 5 min: once at 50 °C, and twice at 65 °C. A 5 μl aliquot of loading dye [0.4 % Xylene Cyanol/0.4 % Bromophenol Blue/1 mM EDTA (pH 8.0)/50 % (v/v) glycerol/0.3 μg/μl ethidium bromide] was added and the RNA was size-fractionated on a 1 % (w/v) agarose gel containing 6.6 % (v/v) formaldehyde, 40 mM Mops, pH 7.0, 10 mM sodium acetate, pH 7.4, 1 mM EDTA, pH 8.0, using as the tank buffer 40 mM Mops, pH 7.0/10 mM sodium acetate, pH 7.4/1 mM EDTA, pH 8.0. The gel was treated for 30 min with 50 mM NaOH, for 30 min with 100 mM Tris/HCl, pH 7.0, and for 40 min with 10 × SSC [where 1 × SSC is 0.15 M NaCl/0.15 M sodium citrate (pH 7.0)]. The RNA was capillary-transferred to a positively charged Nylon membrane (Zetabind, Cuno, Meriden, CT, U.S.A.) in 10 × SSC, and then covalently cross-linked to the membrane with UV light [21]. The cDNA to be used as the probe was ^{32}P-labelled using a random primer labelling kit, following the manufacturer's instructions (Gibco BRL). The unincorporated nucleotides were removed by passing the labelling reaction over a Sephadex G-50 column using 10 mM Tris/HCl, pH 8.0/1 mM EDTA, pH 8.0/100 mM NaCl as the elution buffer and collecting the initial fractions (Pharmacia, Piscataway, NJ, U.S.A.). The probe was boiled for 5 min, before adding 7.5 ml of hybridization solution [0.5 M sodium phosphate (pH 7.2)/7 % (w/w) SDS/1 mM EDTA (pH 8.0)/1 % (w/w) BSA], and then the mixture was incubated with the RNA-containing membrane that had been incubated for 15 min with the hybridization solution alone. After incubation overnight at 61 °C, blots were washed for 4 × 10 min periods in high-stringency buffer [0.04 M sodium phosphate (pH 7.2)/1 mM EDTA (pH 8.0)/1 % (w/v) SDS] at 65 °C, and exposed to film. The results were quantified by densitometry after establishing that the arbitrary absorbance units were within the linear range of the film. Each experiment was repeated at least once with independent batches of cells to document the reproducibility of the results. The cDNA probe used for human AS was the 1842-bp reverse transcriptase–PCR product, described below; the cDNA probe for GRP78 was a 1498-bp sequence between the *Pst*I and *Eco*RI sites [22]. The GH cDNA probe was a 651-bp sequence containing the entire open reading frame [23]. The cDNA probe for the ribosomal protein L7a was a 600-bp sequence between two *Pst*I sites that covered a portion of the coding and untranslated regions within the 3′ half of the full-length cDNA.

Isolation of a human AS cDNA

A cDNA that includes the entire coding sequence for human AS was isolated by reverse transcription of HepG2 mRNA, followed by the reverse transcriptase–PCR procedure. Poly(A)$^+$-selected RNA was isolated from the total RNA by using the polyAttract system from Promega (Madison, WI, U.S.A.). PCR-specific primers (5′-TTGTCGACATCACCCTGACCTGCTTACGCC-3′ and 5′-TTGTCGACGTTCCCCTATCTACCCAC-

AGTCC-3′), containing *Sal*I restriction sites at the 5′-end and designed to amplify the entire coding region of the human AS cDNA (1842 bp), were based on a published sequence [24]. The PCR product was cloned using the TA cloning system from Invitrogen (Carlsbad, CA, U.S.A.). The identity of the AS clone was confirmed by both bidirectional sequencing and comparison with reported sequences [24,25].

Isolation of a human AS genomic promoter fragment

A human AS genomic clone was obtained by PCR-screening of a human PAC genomic library (Genome Systems Inc., St. Louis, MO, U.S.A.) using a set of primers (5′-CAAACCAAGTTC-AGAAGCCTCCC-3′ and 5′-AAGCAGGTCAGGGTGATGT-GGC-3′) to yield a 328-bp sequence covering the proximal promoter region/exon 1 junction, and then re-screening the positive clones with a second set of primers (5′-TGCAATGA-TGGCAAATGCAGCC-3′ and 5′-ACTTGTAGTGGGTCAG-CGTGCGG-3′) covering a 185-bp sequence within exon 12. Primers were designed on the basis of published sequences of the human *AS* gene and cDNA [12,24–26]. Details of the genomic cloning and characterization will be published elsewhere (I. P. Barbosa-Tessmann, C. Zhong and M. S. Kilberg, unpublished work). Based on the 5′-most sequence of a 10.7 kbp *Bam*HI fragment that contained exon 1 and the reported sequence of exon 1 [12,26], we designed specific primers (5′-GGGGATCC-CTGAGTGAACACAATGAACAGA-3′ and 5′-CTGGATCC-TAAGCAGGTCAGGGTGATGTGGCGG-3′) containing restriction sites for *Bam*HI at the 5′-end to PCR-amplify the 3 kbp of the proximal promoter region of the gene. This PCR product was cloned into a TA vector (Invitrogen, San Diego, CA, U.S.A.) and then subcloned as the promoter sequence into a plasmid (pOGH) [23] containing a promoter-less human *GH* gene (Nichols Institute Diagnostics, San Juan Capistrano, CA, U.S.A.) to create the construct named pAS3KbGH.

Transient expression

HepG2 cells were transfected with the AS promoter/GH reporter plasmids described above, as well as a control construct (pMtGH) containing the metallothionein constitutive promoter [27]. We have employed a batch-transfection technique that involves transfection of cells grown to about 75% confluence. The ratio of 10 μg of DNA/60 μl of Superfect reagent (Qiagen, Germany) per 6.6×10^6 cells/100-mm dish was held constant in all transfections. DNA (10 μg) was incubated with 60 μl of Superfect for 10 min at room temperature in MEM without FBS, BSA or antibiotics (as described above) in a total volume of 300 μl. The medium was aspirated, cells were washed once with PBS and transfected for 3 h at 37 °C in a tissue culture incubator in the presence of 5.0 ml of MEM containing FBS, BSA and antibiotics. After transfection, cells were washed once with PBS, culture medium was added, and then the cells were cultured for 24 h. The HepG2 cells on each 100-mm dish were then split between four 60-mm dishes, so that within each experiment cells incubated in either glucose-containing or glucose-free medium arose from the same transfected cell population (this procedure eliminated the concern about transfection efficiency between the fed and starved treatments within a given experiment). After a further 24 h of culture, the cells were incubated for 12 h in appropriate media (see each Figure legend for details). The ability of the AS promoter fragment to promote transcription of the GH reporter gene was evaluated by Northern blot analysis.

RESULTS

Time course of AS mRNA content

To investigate the potential regulation of the human *AS* gene by glucose availability, human HepG2 hepatoma cells were incubated in MEM medium in the presence or absence of 5 mM glucose for 0–24 h before the isolation of RNA for Northern analysis (Figure 1). The human AS cDNA hybridized with an mRNA of approx. 2.0 kb in HepG2 cells. Glucose deprivation induced an increase in AS mRNA content that was detectable after incubation for as little as 8 h, which reached a maximum by 12 h. The AS mRNA content was increased by approx. 16-fold after 12 h of glucose deprivation. The blots were stripped and reprobed with cDNA probes for GRP78 (as a positive control) and the ribosomal protein L7a (as a negative control). As expected, the GRP78 mRNA content also was increased following glucose deprivation, but the time course was slightly

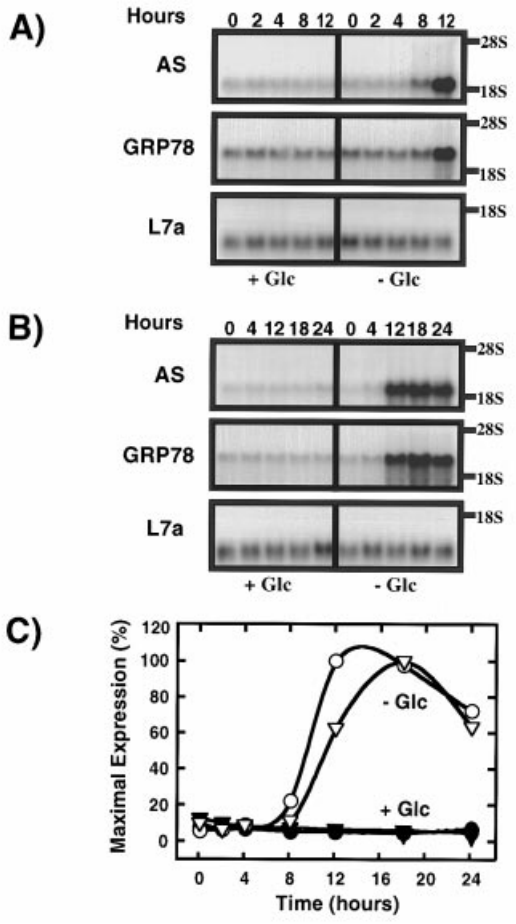

Figure 1 Time course of the change in AS mRNA content in HepG2 cells cultured in the presence or absence of glucose

HepG2 hepatoma cells were cultured in supplemented MEM containing 10% (v/v) FBS and grown to 80% confluence in 60-mm dishes, as described in the Experimental section. At zero time, cells were transferred to glucose-containing MEM (5 mM) or glucose-free MEM, and total RNA was isolated at 0, 2, 4, 8 and 12 h (**A**), or after 0, 4, 12, 18 and 24 h (**B**). Northern blot analysis (20 μg/lane) was performed with a ^{32}P-labelled human AS cDNA probe, as described in the Experimental section; the blot was also probed with a cDNA for GRP78 and the ribosomal protein L7a. The quantified densitometry data for the experiment shown in (**B**) were normalized to those of the experiment in (**A**) using the 12-h data, and then plotted together (**C**) [(○), AS − glucose (Glc); (▽), GRP78 − Glc; (●), AS + Glc; (▼), GRP78 + Glc].

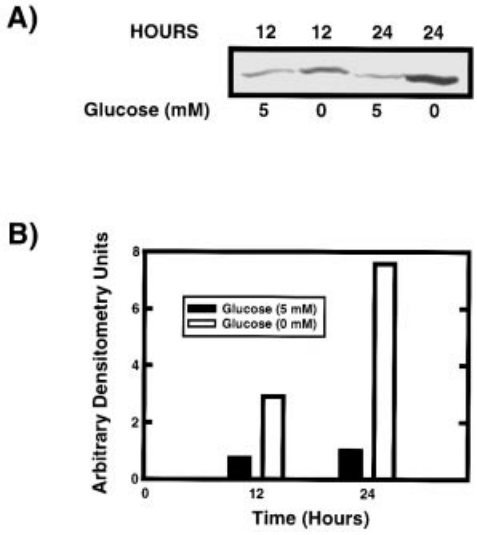

Figure 2 Change in AS protein content of glucose-deprived HepG2 cells

Cells were cultured in MEM containing 10% (v/v) FBS and grown to 80% confluence in 100-mm dishes, as described in the Experimental section. After either a 12-h or 24-h incubation, the cells were transferred to MEM in the presence or absence of glucose at 5 mM. Total cellular protein was extracted by scraping the cells directly into sample dilution buffer, and this was subjected to SDS/PAGE (50 μg of protein/lane) and immunoblot analysis with a monoclonal antibody specific for AS, as described in the Experimental section (12 h values are 0.8 and 2.9 arbitrary densitometry units for 5 and 0 mM glucose respectively; 24 h values are 1.1 and 7.7 units for 5 and 0 mM glucose respectively).

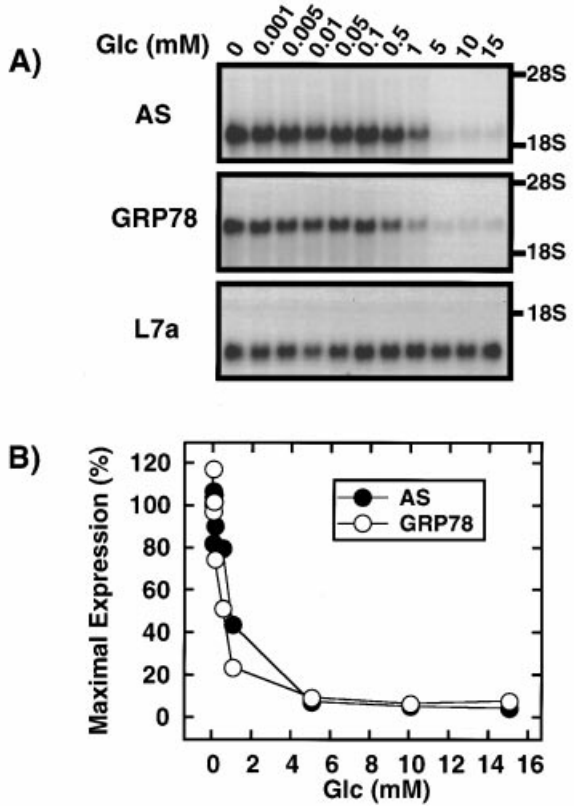

Figure 3 Effect of glucose concentration on AS mRNA content

HepG2 hepatoma cells were cultured in supplemented MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes, as described in the Experimental section. For a 12 h incubation, the cells were transferred to MEM containing glucose at the concentrations indicated. RNA was isolated and subjected to Northern blot analysis (20 μg/lane), as described in the Experimental section. The blots were probed with ^{32}P-labelled cDNA specific for AS, L7a or GRP78 proteins. From a representative experiment, (**A**) shows the autoradiogram and (**B**) represents the data after quantification by densitometry. The variation between two independent experiments was less than 10%. Glc, glucose.

delayed compared with that of AS. The GRP78 mRNA level began to increase at about 12 h, and was maximal at 18 h. In agreement with these results, GRP78 mRNA accumulation was maximal after 16 h in Chinese hamster lung fibroblasts [28]. However, GRP78 protein has been shown not to reach maximum accumulation until 48 h in 3T3-L1 adipocytes [29] or in chick embryo fibroblasts [6]. These data illustrate the responsiveness of the HepG2 cells to glucose availability, and the lack of a change in L7a mRNA level illustrates that the increased content of AS and GRP78 mRNA was a specific response.

AS protein content

To establish whether or not the AS protein content was also increased following glucose deprivation, HepG2 cells were incubated in MEM in the absence or presence of 5 mM glucose for 12 or 24 h (Figure 2). The cells incubated for 12 h in the absence of glucose had 3.6 times the amount of AS protein compared with control cells, whereas after 24 h this increase was even more pronounced (7-fold improvement). As shown previously for amino acid starvation of the *AS* gene [16], the increase in protein lagged temporally behind that of the mRNA, and was not as large relative to control. Nonetheless, the data clearly show that the increased AS mRNA following glucose deprivation is translated into protein.

Glucose concentration

To determine the glucose concentration range over which the AS mRNA content was altered, HepG2 cells were incubated for 12 h in MEM containing glucose concentrations ranging from 0.001 to 15 mM (Figure 3). The responsiveness of the AS mRNA content to glucose concentration qualitatively paralleled that of GRP78, but GRP78 appeared to be relatively slightly more responsive to glucose. The $k_{1/2}$ (concentration causing a half-maximal response) of glucose-dependent repression of the level of AS mRNA was about 1 mM, whereas that of GRP78 mRNA was 0.5 mM. Between 0.5 and 5 mM, the AS mRNA content was proportional to glucose concentration. Our observation that the glucose concentration must be below 1 mM for significant induction of GRP78 to occur is consistent with the results of Kitzman et al. [29]. The absence of an effect on *AS* and *GRP78* gene transcription by the lowest glucose concentrations that were tested might be due to a complete consumption of the glucose before the end of the 12 h incubation period.

Sugar specificity

The specific sugar molecules that cells use to detect the availability of carbohydrate have not been identified conclusively. Data have been reported that point to phosphorylated glucose intermediates, such as glucose 6-phosphate, or metabolites within the pentose phosphate pathway, such as xylulose 5-phosphate, as being putative candidates (reviewed in [3,4]). To investigate the responsiveness of the *AS* gene to the presence of specific carbohydrates, HepG2 cells were incubated for 12 h in MEM

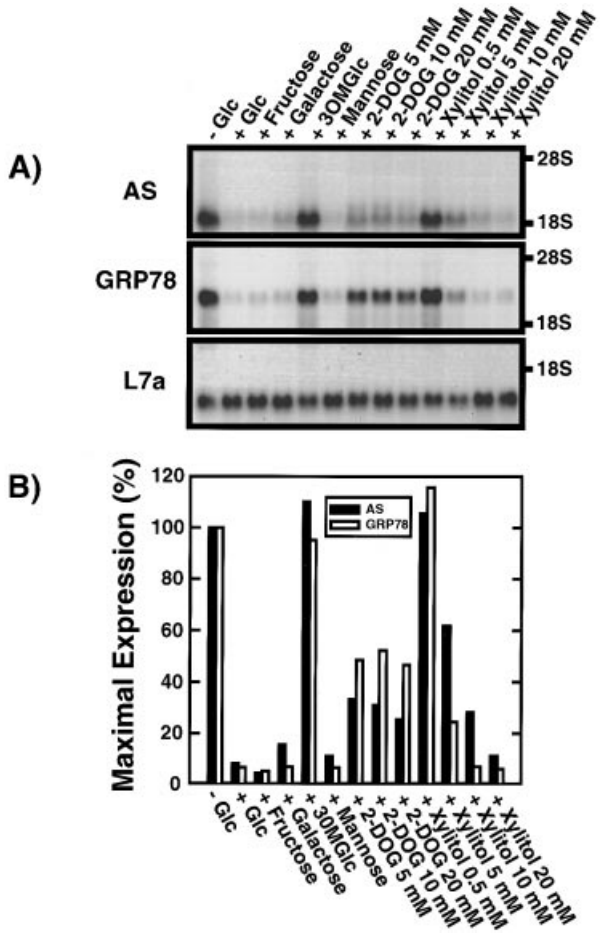

Figure 4 Effect of specific sugar molecules on the content of AS mRNA

HepG2 hepatoma cells were cultured in supplemented MEM containing 10% (v/v) FBS, and grown to 80% confluence in 60-mm dishes, as described in the Experimental section. At zero time, cells were transferred to glucose-free MEM or MEM containing the indicated sugar. The concentration used, where it is not indicated in the Figure, was 5 mM. After a 12 h incubation, RNA was isolated and subjected to Northern blot analysis (20 μg/lane), as described in the Experimental section. The blots were probed with ^{32}P-labelled cDNA specific for AS, L7a or GRP78. From a representative experiment, (**A**) shows the autoradiogram and (**B**) represents the data obtained after quantification by densitometry. Multiple experiments varied by less than 10%.

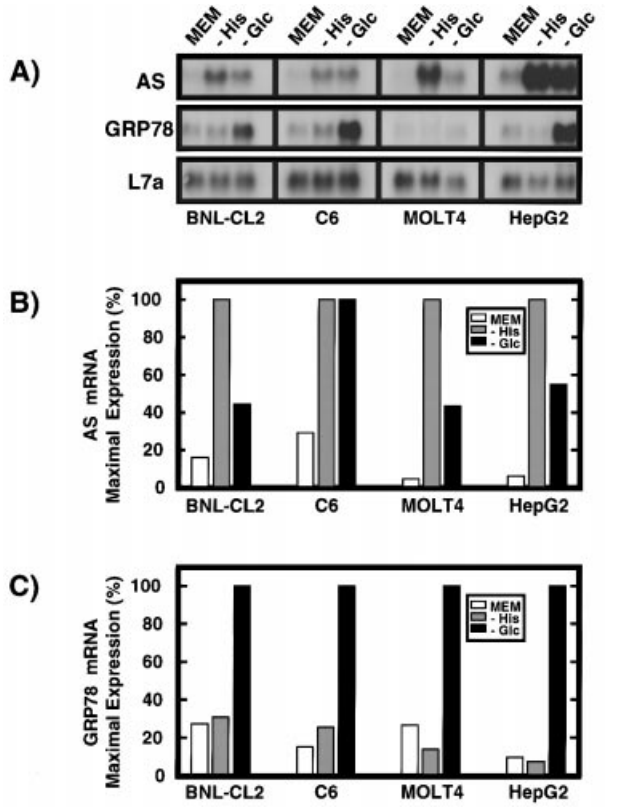

Figure 5 Cell specificity of the amino acid carbohydrate regulation of AS mRNA content

The indicated cell lines were cultured in supplemented MEM containing 10% (v/v) FBS and grown to 80% confluence in 100-mm dishes or T-75 flasks (MOLT4 cells only), as described in the Experimental section. At zero time, cells were transferred to complete MEM, glucose-free MEM or histidine (His)-free MEM (containing 5 mM glucose). After a 12 h incubation, total RNA was isolated and subjected to Northern blot analysis (20 μg/lane), as described in the Experimental section. The blots were probed with ^{32}P-labelled cDNA specific for AS, L7a or GRP78. From a representative experiment, (**A**) shows the autoradiogram and (**B**) and (**C**) represent the data after quantification by densitometry for AS and GRP78 respectively. Glc, glucose.

lacking glucose or MEM supplemented with a variety of sugar metabolites, as shown in Figure 4. As expected from the previous results, AS mRNA content was increased by complete glucose deprivation. The presence of 5 mM each of glucose, fructose, galactose, mannose, DOG, or xylitol prevented, to varying degrees, an increase in the AS mRNA content. Either DOG or xylitol at a concentration of 5 mM resulted in only partial repression of the AS mRNA content. Increasing the concentration of DOG to either 10 or 20 mM resulted in only small additional decreases in the AS mRNA content, whereas there appeared to be a greater response to increasing xylitol concentrations (Figure 4B). At a concentration of 5 mM, the non-metabolizable glucose analogue 3OMGlc did not cause any detectable lowering of the AS mRNA content, relative to that ensuing from complete carbohydrate starvation. These results eliminate free glucose or osmotic changes as being the sensor for carbohydrate availability. Interestingly, GRP78 mRNA was decreased by all of the sugar molecules except 3OMGlc, although the effect of DOG was only partial and concentration-independent between 5 and 20 mM (Figure 4B). The partial repressive effect of DOG is noteworthy, given that it has been reported to increase GRP78 mRNA content in other cell types [30].

Cell specificity

To determine whether or not the nutrient responsiveness of the *AS* gene was species- or cell-type specific, a mouse hepatocyte cell line (BNL-CL2), a rat C6 glioma cell line and a human leukaemic lymphocyte cell line (MOLT4) were compared with HepG2 cells following incubation in complete MEM medium, MEM medium lacking histidine, or MEM medium lacking glucose (Figure 5). The *AS* gene is known to be regulated transcriptionally by the availability of amino acids [14,31] and, as expected, each of the cell lines tested responded to histidine deprivation with an increase in AS mRNA content. Although this response has been documented for a number of cell types and different species, there are no published reports for the four cell lines reported here. Glucose deprivation caused an increase in AS mRNA content in all four cell types tested. These results indicate that the glucose responsiveness of the *AS* gene occurs in both human and

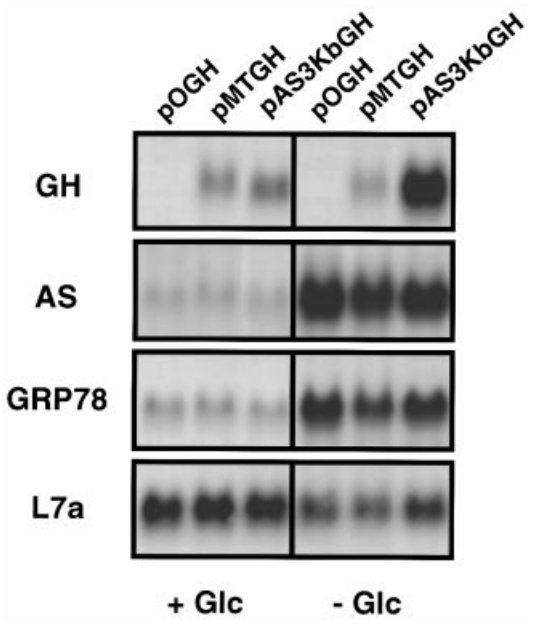

Figure 6 Human AS promoter contains a ChoRE

HepG2 cells were transfected with the indicated construct by the batch method, as described in the Experimental section, to eliminate the problem of possible transfection inefficiency between individual dishes. After incubation in MEM/10% (v/v) dialysed FBS either containing or lacking 5 mM glucose, RNA was isolated and subjected to Northern blot analysis (20 μg/lane). Controls for promoter activity include a *GH* reporter gene construct with no promoter (pOGH) or a constitutive metallothionein promoter (pMTGH). The Northern blot was treated with ^{32}P-labelled probes for GH and endogenous GRP78, L7a and AS, as described in the Experimental section.

rodent cells, and in cells from a variety of tissues. Re-probing the blots with the cDNA for GRP78 confirmed the predicted regulation in each cell type. For both GRP78 and AS, the content of basal and induced mRNAs in the MOLT4 lymphocyte cell line were relatively low (Figure 5).

As already mentioned, it has been reported that, in *Arabidopsis thaliana*, the *ASN1* gene is repressed by carbohydrate, but this repression is partially relieved by supplementation of the medium with amino acids [17]. The increase in AS mRNA content in HepG2 cells incubated in MEM lacking glucose, but containing a full complement of amino acids (Figure 5), illustrates that for the mammalian gene it would appear that both amino acid and glucose deprivation cause up-regulation of the gene. Likewise, histidine deprivation enhances the AS mRNA content, despite the presence of 5 mM glucose (Figure 5).

A carbohydrate response element (ChoRE) exists in the AS promoter

The transcriptional activity of the *AS* gene is regulated by amino acid availability, and thereby alters the cellular content of both AS mRNA and protein [14,31]. To determine if the changes in AS mRNA content in response to glucose deprivation involve the transcriptional control of the gene, we isolated the proximal 3 kbp of the human gene promoter region, and subcloned it in front of a reporter construct containing the human *GH* gene lacking a promoter. Following transient transfection, the cells were subjected to a 12 h incubation in MEM either containing or lacking glucose, and Northern blot analysis was used to investigate the GH mRNA content. Endogenous levels of AS and GRP78 mRNA served as a positive control. No GH mRNA was produced after transfection of the promoter-less construct pOGH (Figure 6). Using a metallothionein promoter that should have yielded constitutive expression resulted in detectable GH mRNA but, as expected, glucose deprivation had no stimulatory effect. The 3 kb promoter sequence of the *AS* gene was able to promote specific transcription of the *GH* gene under amino-acid-fed conditions. Furthermore, when cells transfected with the AS promoter sequence were incubated in glucose-free medium, the GH mRNA content was increased (Figure 6). The degree of induction of the GH mRNA was as great as that observed for the endogenous AS or GRP78 mRNA levels; therefore the *cis*-acting regulatory element(s) necessary for transcriptional regulation of the *AS* gene by glucose availability are present in the proximal 3 kb promoter sequence.

DISCUSSION

Mammalian cells respond to deprivation of essential amino acids by increasing the transcription of the *AS* gene, which results in a subsequent elevation in the steady-state levels of AS mRNA, protein and enzymic activities [11,14–16,31]. The data in the present study document that there is also an increased transcription of the *AS* gene in response to carbohydrate limitation. When the results of many experiments were averaged, it was shown that an induction of approx. 14-fold in the steady-state AS mRNA content occurs 12 h after removal of glucose from the culture medium. This time course is similar to that observed following amino acid deprivation in rat hepatoma cells [15], but preceded the maximal increase in mRNA for the glucose-regulated protein GRP78, for which the mRNA was maximally elevated after 18 h. The AS protein content also increased following the rise in mRNA. These results confirm that the *AS* gene should be added to a family of glucose-responsive genes, some of which are down-regulated by glucose deprivation [3–5], whereas others are elevated by the lack of glucose. Interestingly, documented examples of the latter are limited, but include the molecular chaperones GRP78 and GRP98 [6] and the GLUT1 [7,8] and GLUT3 transporters [9].

There are several possible physiological reasons for induction of *AS* expression in response to carbohydrate deprivation. In plants, for example, it has been proposed that because the carbon:nitrogen ratio of asparagine is less than that for glutamine, plants switch to asparagine as the primary form of nitrogen transport and storage during carbohydrate limitation [18,32]. In maize root tips, AS enzymic activity was responsible for a 27-fold increase in asparagine content following glucose starvation [33] and increases in steady-state AS mRNA content have been observed [18]. In *A. thaliana*, a glutamine-dependent AS mRNA (ASN1) accumulated in plants grown under dark conditions [17], i.e. a condition in which carbohydrate synthesis is reduced. This dark-induced AS mRNA accumulation was repressed by sucrose, but the repression was partially circumvented by amino acids. These findings were interpreted to indicate that the *ASN1* gene is controlled by the nitrogen:carbon ratio [17]. Similar mechanisms might exist in animals. Future experimentation using an *in vivo* model of carbohydrate limitation will be required to fully test this hypothesis, and to investigate asparagine synthesis and utilization by specific tissues.

Carbohydrate limitation and the resulting energetic stress might also initiate asparagine biosynthesis, because this amino acid possesses an important, but not yet fully understood, role in cell growth control. It is interesting to note that AS was uniquely able to complement the cell cycle mutant studied by Greco et al. [12]. Furthermore, asparagine deprivation via treatment of cells

with asparaginase induces apoptosis [34]. One may speculate whether asparagine limitation alters progression through the cell cycle and induction of programmed cell death solely by its decreased availability for protein synthesis, or whether this amino acid serves another role, perhaps as a signal molecule. If so, both carbohydrate and amino acid starvation might trigger asparagine biosynthesis, which could act as an indicator of insufficient substrate for continued cell division; research is currently in progress to test this hypothesis.

The glucose analogue, 3OMGlc, which is transported into cells but cannot be phosphorylated by hexokinases, was unable to repress the induction of either AS or GRP78 in the absence of glucose. Likewise, it has been reported by others [35] that 3OMGlc cannot mimic the effect of glucose for the carbohydrate-induced genes encoding fatty acid synthase and acetyl-CoA carboxylase. These data suggest that free glucose is not the signal molecule, and that further metabolism is therefore necessary for the transcriptional regulation of genes that are either induced or repressed by the presence of carbohydrate. Our data illustrate that fructose, galactose or mannose (all at 5 mM) repressed the induction of AS and GRP78 under conditions of glucose starvation. These sugars have been reported to alter gene expression in other situations as well; for example, fructose enhanced transcription of the L-type pyruvate kinase [36]. Although mRNA content was not commented upon by Kitzman et al. [29], these authors reported that galactose did not repress the protein content of GRP78 in 3T3-L1 adipocytes cultured in the absence of glucose. The lack of an effect by galactose might be due to differences in galactose metabolism between HepG2 hepatoma cells and 3T3-L1 adipocytes, or perhaps changes in mRNA occurred in the 3T3-L1 cells that were not reflected in altered protein levels. In agreement with our results indicating that mannose can effectively replace glucose in the carbohydrate-sensing pathway, this sugar was shown to induce the expression of acetyl-CoA carboxylase in adipose tissue of suckling rats, although both fructose and galactose failed to elicit an effect [37].

Fructose, galactose and mannose can be phosphorylated and subsequently converted into glucose 6-phosphate. Published evidence supports the proposal that glucose 6-phosphate can serve as a signal molecule in regulating the expression of the sugar-induced genes (reviewed in [3]). The glucose analogue DOG is phosphorylated, but in most tissues its further metabolism is poor, which leads to an accumulation of DOG 6-phosphate [38]. As a result, DOG has been used as a substitute for glucose 6-phosphate, which is not transported effectively into mammalian cells. We observed in HepG2 cells that 20 mM DOG could partly prevent the enhanced steady-state content of either AS or GRP78 mRNA, but could not maintain them at the basal levels achieved by 5 mM glucose. However, in the liver, it has been reported that DOG 6-phosphate is not the only end product, i.e. further hepatic metabolism of DOG 6-phosphate does occur, in contrast with that found in other tissues [38,39]. It might be possible that HepG2 hepatoma cells retain this characteristic of liver metabolism, and DOG is not a definitive test for the role of glucose 6-phosphate as a repressor of *AS* and *GRP78* gene transcription in these cells.

It has been suggested that metabolites of the pentose phosphate pathway might also serve as signals of sugar availability [3]. Doiron et al. [40] showed that xylitol increased expression of a reporter gene driven by the L-type pyruvate kinase promoter; however, experiments monitoring the xylitol-induced accumulation of fatty-acid synthase mRNA have correlated the response with the cellular level of glucose 6-phosphate, but not with the content of xylulose 5-phosphate or glycolytic metabolites other than glucose 6-phosphate [41]. As shown above, in HepG2 cells xylitol can prevent the induction of either the *AS* or the *GRP78* gene in the absence of glucose. Our results thus far indicate that glucose must be phosphorylated in order to at least signal the transcriptional repression of both *AS* and *GRP78* in carbohydrate-fed conditions, but further experimentation will be required to firmly establish the specific sugar metabolite(s) responsible for *AS* gene repression.

Glucose-regulated proteins were first identified following transformation of fibroblasts with avian sarcoma virus [42,43], and Shiu et al. [6] reported that the viral-dependent increase of these proteins was due to depletion of glucose from the medium by the transformed cells. In addition to glucose starvation, several other stress conditions can induce glucose-regulated-protein gene expression. The glucose-regulated proteins are believed to be chaperones which bind to misfolded proteins, and thus prevent aggregation in the endoplasmic reticulum [44]. The two most common of these are GRP78 and GRP94, with GRP78 being the most abundant [45]. Our results add several cell lines to the list of those reported to respond to glucose deprivation with an increase in GRP78 mRNA content, and indicate that the expression of GRP78 mRNA is unaltered by amino acid limitation in human HepG2 cells. The effect of the amino acid analogue azetidine 2-carboxylate on GRP78 expression was studied in HeLa cells [46]. In contrast with histidine starvation, GRP78 protein and mRNA levels were increased after 4 h of treatment with 5 mM azetidine 2-carboxylate. This analogue of proline is incorporated into elongating polypeptides, resulting in abnormal protein folding that is detected by the chaperone GRP78 [46]. These results demonstrate that the content of GRP78 mRNA is altered differently in response to synthesis of abnormal proteins, as opposed to amino acid deprivation with a possible concurrent suppression of total protein synthesis.

The molecular mechanisms that mediate nutrient control of gene expression in mammalian cells are not well understood. Identification of the human AS gene as one that is transcriptionally regulated by the availability of both amino acids and carbohydrates represents a unique opportunity for investigation. The metabolic signals that initiate the induction of *AS* gene expression remain to be elucidated, as do the resulting metabolic consequences of such changes. The observation that asparagine limitation prevents progression through the cell cycle [12,13], which is selectively toxic to certain tumour cells [16], underscores the importance of future studies on nutrient control of the *AS* gene.

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REFERENCES

1 Clarke, S.D. and Abraham, S. (1992) FASEB J. **6**, 3146–3152
2 Kilberg, M. S., Hutson, R. G. and Laine, R. O. (1994) FASEB J. **8**, 13–19
3 Girard, J., Ferré, P. and Foufelle, F. (1997) Annu. Rev. Nutr. **17**, 325–352
4 Towle, H. C., Kaytor, E. N. and Shih, H.-M. (1997) Annu. Rev. Nutr. **17**, 405–433
5 Waeber, G., Thompson, N., Haefliger, J.-A. and Nicod, P. (1994) J. Biol. Chem. **269**, 26912–26919
6 Shiu, R. P. C., Pouyssegur, J. and Pastan, I. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3840–3844
7 Kitzman, Jr., H. H., McMahon, R. J., Williams, M. G. and Frost, S. C. (1993) J. Biol. Chem. **268**, 1320–1325

8 Wertheimer, E., Sasson, S., Cerasi, E. and Ben-Neriah, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2525–2529
9 Nagamatsu, S., Sawa, H., Inoue, N., Nakamichi, Y., Takeshima, H. and Hoshino, T. (1994) Biochem. J. **300**, 125–131
10 Richards, N. G. J. and Schuster, S. M. (1998) Adv. Enzymol. **72**, 145–198
11 Arfin, S. M., Simpson, D. R., Chiang, C. S., Andrulis, I. L. and Hatfield, G. W. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2367–2369
12 Greco, A., Gong, S. S., Ittmann, M. and Basilico, C. (1989) Mol. Cell. Biol. **9**, 2350–2359
13 Gong, S. S. and Basilico, C. (1990) Nucleic Acids Res. **18**, 3509–3513
14 Gong, S. S., Guerrini, L. and Basilico, C. (1991) Mol. Cell. Biol. **11**, 6059–6066
15 Hutson, R. G. and Kilberg, M. S. (1994) Biochem. J. **303**, 745–750
16 Hutson, R. G., Kitoh, T., Amador, D. A. M., Cosic, S., Schuster, S. M. and Kilberg, M. S. (1997) Am. J. Physiol. **272**, C1691–C1699
17 Lam, H.-M., Peng, S. S. Y. and Coruzzi, G. M. (1994) Plant Physiol. **106**, 1347–1357
18 Chevalier, C., Bourgeois, E., Just, D. and Raymond, P. (1996) Plant J. **9**, 1–11
19 Eagle, H. (1955) Science **122**, 501–504
20 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
21 Church, G. M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1991–1995
22 Lee, A. S., Delegeane, A. and Scharff, D. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4922–4925
23 Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. and Moore, D. D. (1986) Mol. Cell. Biol. **6**, 3173–3179
24 Andrulis, I. L., Chen, J. and Ray, P. N. (1987) Mol. Cell. Biol. **7**, 2435–2443
25 Greco, A., Ittmann, M. and Basilico, C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1565–1569
26 Zhang, Y. P., Lambert, M. A., Cairney, A. E. L., Wills, D., Ray, P. N. and Andrulis, I. L. (1989) Genomics **4**, 259–265
27 Hamer, D. H. and Walling, M. (1982) J. Mol. Appl. Genet. **1**, 273–288
28 Lin, A. Y. and Lee, A. S. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 988–992
29 Kitzman, Jr., H. H., McMahon, R. J., Aslanian, A. M., Fadia, P. M. and Frost, S. C. (1996) Mol. Cell. Biochem. **162**, 51–58
30 Pouysségur, J., Shiu, R. P. C. and Pastan, I. (1977) Cell **11**, 941–947
31 Guerrini, L., Gong, S. S., Mangasarian, K. and Basilico, C. (1993) Mol. Cell. Biol. **13**, 3202–3212
32 Tsai, F.-Y. and Coruzzi, G. M. (1990) EMBO J. **9**, 323–332
33 Brouquisse, R., James, F., Raymond, P. and Pradet, A. (1991) Plant Physiol. **96**, 619–626
34 Story, M. D., Voehringer, D. W., Stephens, L. C. and Meyn, R. E. (1993) Cancer Chem. Pharm. **32**, 129–133
35 Foufelle, F., Gouhot, B., Pégorier, J.-P., Perdereau, D., Girard, J. and Ferré, P. (1992) J. Biol. Chem. **267**, 20543–20546
36 Munnich, A., Lyonnet, S., Chauvet, D., Van Schaftingen, E. and Kahn, A. (1987) J. Biol. Chem. **262**, 17065–17071
37 Brun, T., Roche, E., Kim, K.-H. and Prentki, M. (1993) J. Biol. Chem. **268**, 18905–18911
38 Jenkins, A. B., Furler, S. M. and Kraegen, E. W. (1986) Int. J. Biochem. **18**, 311–318
39 Rencurel, F., Waeber, G., Antoine, B., Rocchiccioli, F., Maulard, P. and Girard, J. (1996) Biochem. J. **314**, 903–909
40 Doiron, B., Cuif, M.-H., Chen, R. and Kahn, A. (1996) J. Biol. Chem. **271**, 5321–5324
41 Mourrieras, F., Foufelle, F., Foretz, M., Morin, J., Bouche, S. and Ferre, P. (1997) Biochem. J. **326**, 345–349
42 Stone, K. R., Smith, R. E. and Joklik, W. K. (1974) Virology **58**, 86–100
43 Isaka, T., Yoshida, M., Owada, M. and Toyoshima, K. (1975) Virology **65**, 226–237
44 Pelham, H. R. B. (1986) Cell **46**, 959–961
45 Lee, A. S. (1992) Curr. Opin. Cell Biol. **4**, 267–273
46 Watowich, S. S. and Morimoto, R. I. (1988) Mol. Cell Biol. **8**, 393–405

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