cAMP prevents the glucose-mediated stimulation of GLUT2 gene transcription in hepatocytes

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Glucose homoeostasis necessitates the presence in the liver of the high K_m glucose transporter GLUT2. In hepatocytes, we and others have demonstrated that glucose stimulates GLUT2 gene expression *in vivo* and *in vitro*. This effect is transcriptionally regulated and requires glucose metabolism within the hepatocytes. In this report, we further characterized the *cis*-elements of the murine GLUT2 promoter, which confers glucose responsiveness on a reporter gene coding the chloramphenicol acetyl transferase (CAT) gene. 5′-Deletions of the murine GLUT2 promoter linked to the CAT reporter gene were transfected into a GLUT2 expressing hepatoma cell line (mhAT3F) and into primary cultured rat hepatocytes, and subsequently incubated at low and high glucose concentrations. Glucose stimulates gene transcription in a manner similar to that observed for the endogenous GLUT2 mRNA in both cell types; the −1308 to −212 bp region of the promoter contains the glucose-responsive elements. Furthermore, the −1308 to −338 bp region of the promoter contains repressor elements when tested in an heterologous thymidine kinase promoter. The glucose-induced GLUT2 mRNA accumulation was decreased by dibutyryl-cAMP both in mhAT3F cells and in primary hepatocytes. A putative cAMP-responsive element (CRE) is localized at the −1074/−1068 bp region of the promoter. The inhibitory effect of cAMP on GLUT2 gene expression was observed in hepatocytes transfected with constructs containing this CRE (−1308/+49 bp fragment), as well as with constructs not containing the consensus CRE (−312/+49 bp fragment). This suggests that the inhibitory effect of cAMP is not mediated by the putative binding site located in the repressor fragment of the GLUT2 promoter. Taken together, these data demonstrate that the elements conferring glucose and cAMP responsiveness on the GLUT2 gene are located within the −312/+49 region of the promoter.

INTRODUCTION

The high K_m glucose transporter GLUT2 is expressed in liver, kidney, small intestine, some restricted areas of the brain and in β cells of the endocrine pancreas [1].

In vivo, the expression of liver GLUT2 is increased by hyperglycaemia in both control [2] and diabetic rats [3,4]. GLUT2 expression is also stimulated by high glucose concentrations in primary cultures of rat hepatocytes [2,5], and glucose metabolism is required to confer glucose responsiveness [6]. As observed in liver, GLUT2 expression is increased by high glucose concentrations *in vitro* in β pancreatic cells [7,8], and glucose metabolism is required for this regulation [9]. This effect is transcriptionally regulated and no stabilization of GLUT2 mRNA by glucose is observed in INS-1 cells [6,10]. Despite hyperglycaemia, GLUT2 gene expression is paradoxically decreased in the β cells of several experimental models of diabetes [11–15]. The discrepancy between liver and pancreatic β cells in their response to diabetic hyperglycaemia could be due to tissue-specific factors [13,16].

A detailed analysis of the GLUT2 promoter has been performed in the insulinoma cell lines INS-1 and βTC3 [17,18]. In INS-1 cells, the chloramphenicol acetyl transferase (CAT) activity driven by a fragment of the human GLUT2 promoter was shown to be stimulated by glucose in a dose-dependent manner [10]. It was also shown that the −338/+49 bp region of the GLUT2 promoter contains sequences responsible for pancreatic islet and liver GLUT2 expression *in vivo* and *in vitro* [17,18]. Taking into account the differences in the regulation of GLUT2 expression in liver and pancreatic β cells during diabetes, it was of interest to determine the elements involved in the transcriptional regulation of GLUT2 expression in response to glucose, both in cultured hepatocytes and in a hepatoma cell line which expresses GLUT2.

As many key enzymes present in the liver are regulated by the activation of the cAMP–protein kinase A cascade, secondarily to the binding of glucagon to its receptor, we studied the potential role of cAMP in the control of GLUT2 mRNA expression and gene transcription. A potential site for a cAMP responsive element (CRE) was localized previously −1074 bp upstream of the start site in the promoter of the GLUT2 gene [10], but so far no functional data have been described.

The aim of the present study was to characterize the regions of the GLUT2 promoter gene which are involved in the stimulatory effect of glucose and the regulation by cAMP of liver GLUT2 gene expression.

METHODS

Primary culture of hepatocytes

Female Wistar rats (200–220 g) housed at 24 °C with light from 07:00 h to 19:00 h were used. Hepatocytes were isolated by the

Abbreviations used: GLUT2, glucose transporter 2; CAT, chloramphenicol acetyl transferase; CRE, cAMP-responsive element; FCS, fetal calf serum; L-PK, L-pyruvate kinase; tk, thymidine kinase.

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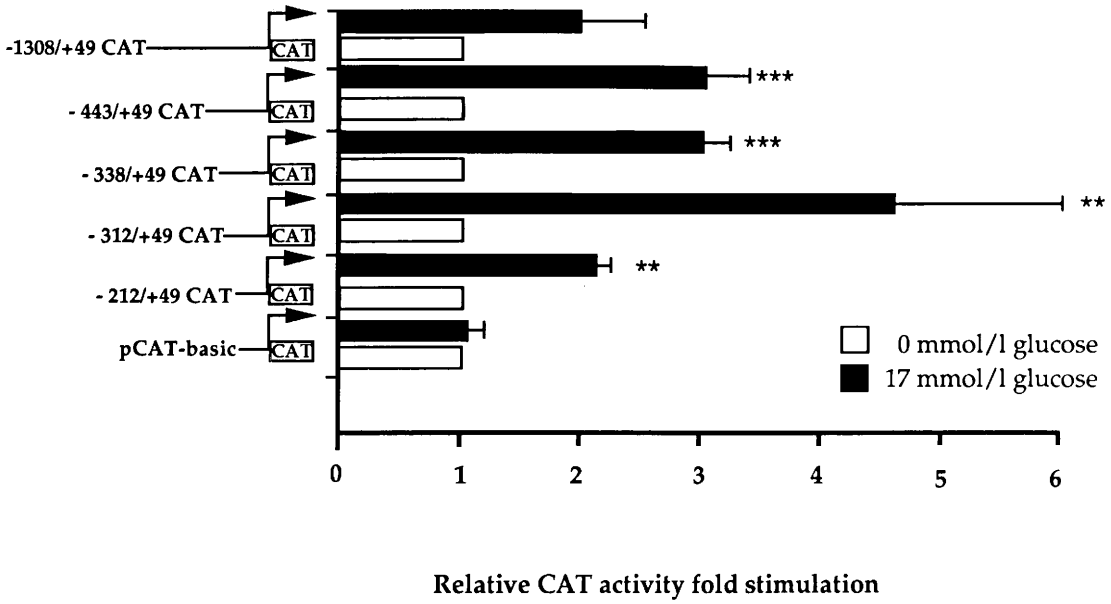

Figure 1 5′-Deletion study of the murine GLUT2 promoter linked to the CAT reporter gene transfected into mhAT3F cells: relative CAT activity in the presence of glucose

Cells were transfected by lipofection with 10 μg of plasmid/dish, as described in the Methods section. After overnight culture, the medium containing liposomes was replaced by fresh medium with (17 mmol/l) or without glucose. Cells were harvested 36 h after replacement of the medium. The CAT activity, assayed in 40 μg of cellular proteins, was calculated as the percentage of acetylated chloramphenicol versus non-acetylated chloramphenicol. The effect of glucose was expressed as fold stimulation over the CAT activity measured in cells cultured in the absence of glucose. pCAT-basic is the promoterless vector coupled to the CAT reporter gene; RSV–CAT is the Rous sarcoma virus enhancer coupled to the CAT reporter gene. Results are means ± S.E.M. of seven experiments; $^*P < 0.01$ when compared with CAT activity measured in cells cultured in the absence of glucose.

method of Berry and Friend [19] from rats in the post-absorptive period, i.e. at 09:00 h. Dissociation of the cells was performed in Hepes buffer (137 mmol/l NaCl/2.7 mmol/l KCl/0.7 mmol/l Na_2HPO_4,12 H_2O/10 mmol/l Hepes, pH 7.5 at 37 °C) containing 0.1 % (w/v) collagenase (1.36 units/mg; Boehringer Mannheim, Meylan, France) and 5 mmol/l $CaCl_2$. For each hepatocyte preparation, the cell viability estimated by Trypan Blue exclusion was > 90 %.

Hepatocytes were suspended in medium 199 containing Earle's salts, 2.2 mg/l $NaHCO_3$ (Gibco-BRL, Cergy Pontoise, France) with penicillin (10 units/ml), streptomycin (100 mg/ml) and kanamycin (50 mg/ml), and supplemented with fetal calf serum (FCS; 10 %, v/v) (Jacques Boy, Reims, France). Hepatocytes were plated in 100-mm plastic dishes [$(8–10) \times 10^6$ cells/dish]. For attachment, cells were cultured for 4 h in medium 199 to which were added 10 % (v/v) FCS, 1 nmol/l insulin (Actrapid, Novo, Copenhagen, Denmark), 100 nmol/l dexamethasone (Soludecadron, Merck & Co., Riom, France), 2.2 mmol/l glutamine (Gibco-BRL) and 0.05 % (v/v) serum albumin. The medium was then replaced by fresh medium 199 containing only 5 % (v/v) FCS. We used 5 % FCS in the culture medium, since in preliminary experiments we showed that FCS allowed GLUT2 mRNA concentration to be maintained, for at least 48 h, at a level comparable to that observed in liver before cell dissociation (results not shown).

When the cultures were performed in the absence of glucose, 10 mmol/l lactate and 1 mmol/l pyruvate were added as oxidative substrates. Nevertheless, when the cells were cultured in the absence of glucose in the medium, hepatocytes could produce glucose from lactate, pyruvate and amino acids present in the culture medium, and glucose concentration in the culture medium was about 1 mmol/l after 24 h of culture (results not shown).

To study the effect of cAMP, cells were placed, immediately after the attachment period, in 100 μmol/l dibutyryl-cAMP, protected from degradation by 5 mmol/l theophylline, for 24 h.

Transient transfection studies

For transient transfection studies, the hepatocytes were suspended in PBS buffer (137 mmol/l NaCl/1.3 mmol/l KCl/16.1 mmol/l Na_2HPO_4, 12 H_2O/1.5 mmol/l KH_2PO_4) containing 10 % (v/v) FCS at a concentration of 24×10^6 cells/ml and electroporated.

An aliquot (750 μl) of cells was suspended in electroporation cuvettes (0.4 cm in depth; Bio-Rad, Ivry/Seine, France) containing 40 μg of plasmid and 400 μg of deproteinized and sonicated salmon-sperm DNA split into four dishes. Electroporation was performed using a gene pulser (Bio-Rad) at a capacitance of 960 μF and 250 mV. After electroporation cells were immediately resuspended in 1 ml of attachment medium [medium 199 with the addition of 10 % (v/v) FCS, 1 nmol/l insulin (Actrapid, Novo), 10 μmol/l dexamethasone (Soludecadron, Merck & Co.) 2.2 mmol/l glutamine (Gibco-BRL) and 0.05 % (v/v) serum albumin] and cells from one cuvette were seeded in four dishes (60 mm diameter) containing 4 ml of the same medium. After an attachment period of 4 h, medium was replaced by fresh medium supplemented with 5 % (v/v) FCS with or without 20 mmol/l of glucose.

After 24 h or 40 h in culture, cells were scraped and ruptured in 400 μl/dish of transfection lysis buffer (Promega, COGER, Paris, France). After removal of cellular debris, the extracts were heated for 10 min at 65 °C to destroy endogenous deacetylating activity. The CAT activity was assayed in 100 μg of total protein, chloramphenicol and its acetylated products were separated after 2 h on TLC plates (Merck & Co.), the plates were exposed for 2 days (Hyperfilm, Amersham, Les Ulis, France) and quantified by

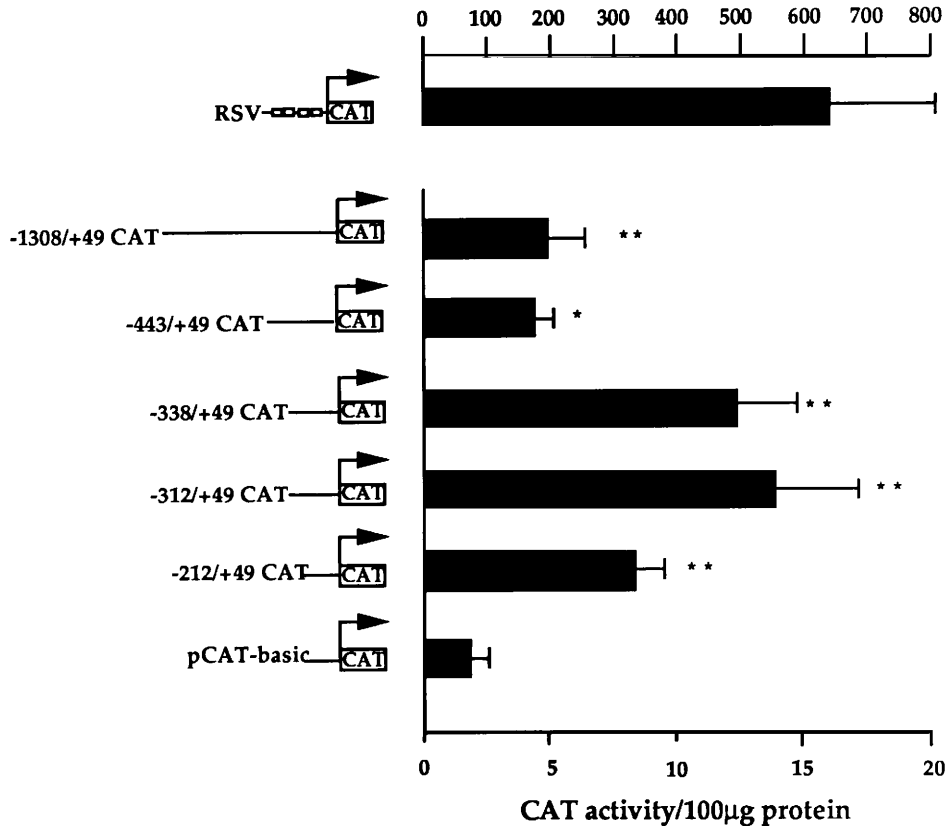

Figure 2 Relative CAT activity of the GLUT2 promoter in a primary culture of hepatocytes in the presence of glucose

Cells were transfected by electroporation with 10 μg of plasmid/dish, as described in the Methods section. After a 4 h attachment period, the culture medium was replaced by fresh medium supplemented with 20 mmol/l glucose. Cells were harvested after 24 h. Results are expressed as CAT activity assayed in 100 μg of cellular proteins and are calculated as the percentage of the acetylated form with respect to non-metabolized chloramphenicol. Quantification of the CAT activity of different fragments of the GLUT2 promoter coupled to the CAT reporter gene, in hepatocytes cultured in the presence of 20 mmol/l of glucose, was carried out. The results are means $\pm$ S.E.M. of five experiments; $^{*}P < 0.05$, $^{**}P < 0.001$ when compared with pCAT-basic (the promoterless vector coupled to the CAT reporter gene) activity.

liquid scintillation counting. The CAT activity was calculated as the ratio of the acetylated forms to the sum of acetylated plus non-acetylated forms of chloramphenicol per μg of cell protein. The effect of glucose was then expressed as fold stimulation over the CAT activity measured in cells cultured in the absence of glucose.

Hepatoma cell culture and transfection

The mhAT3F hepatocyte cell line was derived from the tumourous liver of a transgenic mouse expressing simian virus 40 early genes under the control of the liver-specific antithrombin III promoter [20]. Cells were grown in Dulbecco's modified Eagle's medium/Ham F12 glutamax medium (Gibco-BRL) supplemented with 100 nmol/l insulin (Novo), 1 μmol/l dexamethasone (Soludecadron, Merck & Co.), 1 μmol/l tri-iodothyronine (Sigma, St. Louis, MO, U.S.A.), 30 nmol/l $Na_2S_2O_3$ (Sigma, St. Quentin Fallavier, France) and 5% (v/v) FCS (Jacque Boy).

To study the effect of cAMP, cells were placed in 100 μmol/l dibutyryl-cAMP, protected from degradation by 5 mmol/l theophylline for 24 h, with or without glucose after an overnight in the absence of glucose.

The mhAT3F cells were transfected with lipofection {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulphate (DOTAP); Boehringer Mannheim}, as recommended by the manufacturer. When cells were at 60% of confluency, they were maintained for 6 h in Dulbecco's modified Eagle's medium/F12 (Gibco-BRL) in the absence of glucose and insulin. Cationic liposomes (30 μg/60 mm dish) (DOTAP; Boehringer Mannheim) were incubated for 15 min with 10 μg of GLUT2/CAT plasmids in 100 μl of Hepes-buffered saline (20 mmol/l Hepes/150 mmol/l NaCl). The liposome–DNA complexes were added to mhAT3F cells cultured without glucose in a medium deprived of FCS. After overnight culture, the medium containing liposomes was replaced by fresh medium supplemented only with 5% (v/v) FCS with or without glucose. Cells were harvested 36 h after removal of the liposomes. CAT activity was assayed on 40 μg of total protein as described previously for transfected hepatocytes.

Quantification of GLUT2 mRNA concentration

Total RNA was isolated by the method of Chomczynski and Sacchi [21]. The Northern blot and hybridization were performed with 20 μg of total RNA as described previously [2]. The GLUT2 cDNA probe was kindly supplied by Dr. B. Thorens (Institute of Pharmacology and Toxicology, University of Lausanne, Switzerland) [1].

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed by using the Wilcoxon test for unpaired data.

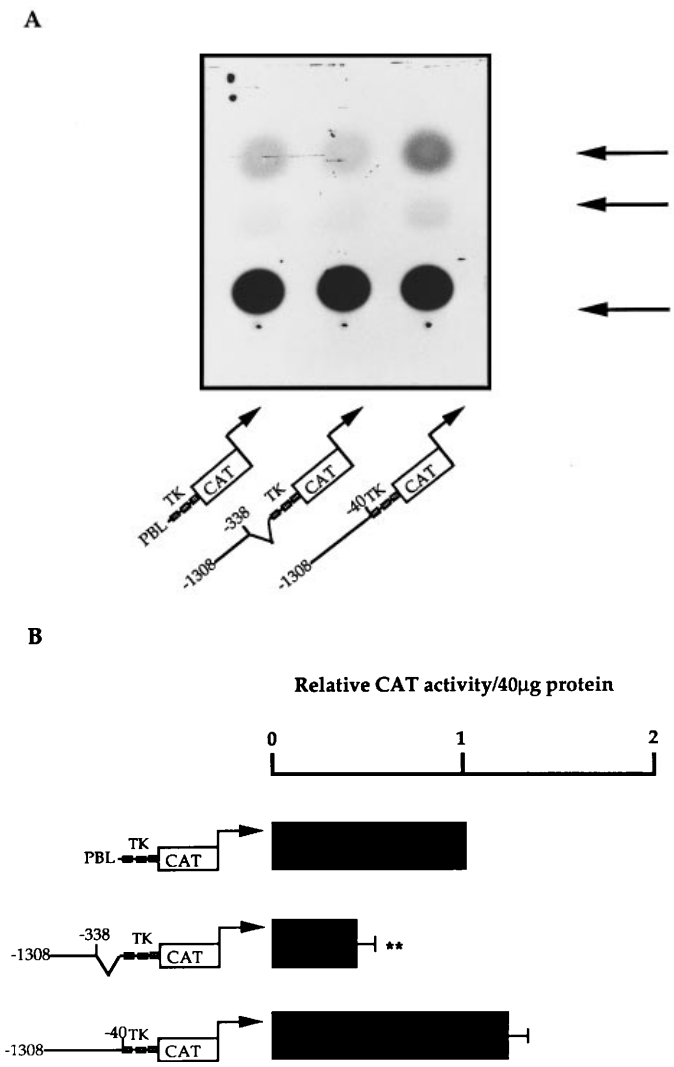

Figure 3 Evidence for the presence of repressor element(s) between −1308 and −338 bp of the GLUT2 promoter in the hepatoma cell line mhAT3F cultured in the presence of glucose

Plasmids (10 μg), containing GLUT2 promoter sequences from −1308 to −40 and from −1308 to −338 linked to a minimal thymidine kinase tkCAT promoter, were transfected in hepatoma mhAT3F cells by lipofection, as described in the Methods section. After overnight culture, the medium containing the liposomes was replaced by fresh medium containing 17 mmol/l glucose. Cells were harvested 36 h after removal of the liposomes. CAT activity was assayed in 40 μg of total cellular extracts as described in the Methods section. (**A**) A representative thin-layer chromatograph showing mono- and tri-acetylated and non-acetylated forms of chloramphenicol designated by arrows. (**B**) CAT activity was calculated as the percentage of acetylated form relative to non-metabolized chloramphenicol per 40 μg of cell protein. CAT activity was compared with that measured in cells transfected with PBL-tk CAT (the 5′-region of the minimal tk promoter fused to a CAT reporter gene) and cultured in the presence of 17 mmol/l glucose, and used as standard. Results are means ± S.E.M. of six different experiments; $^{**}P < 0.005$ when compared with PBL-tk-CAT.

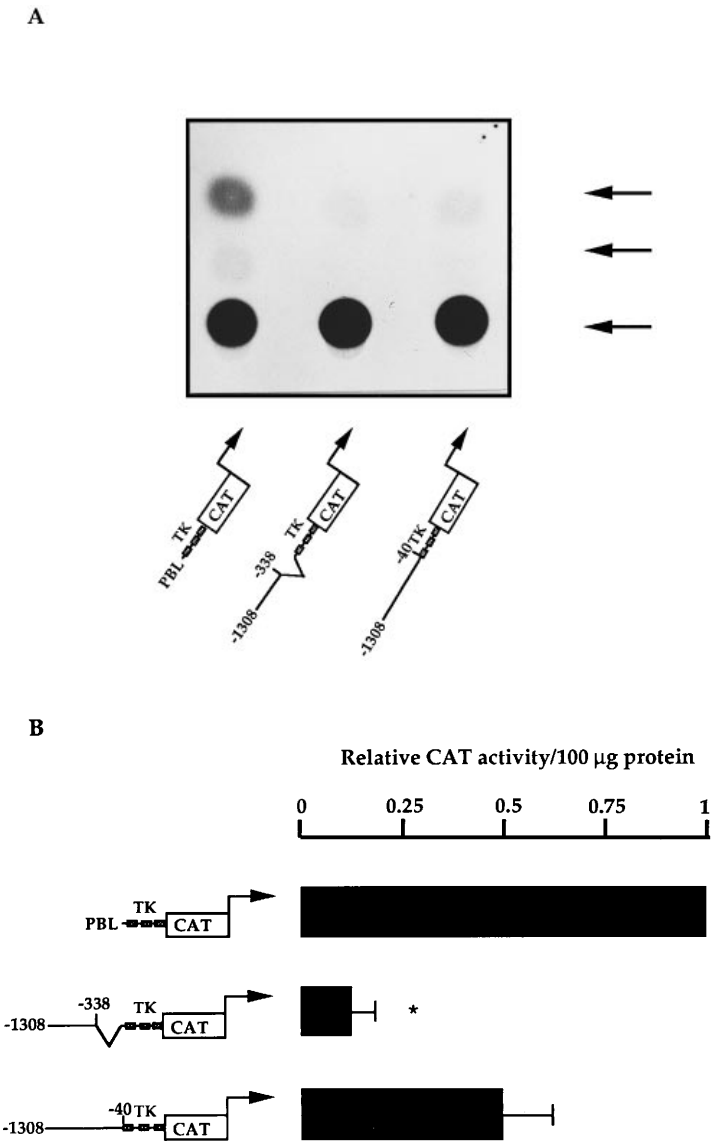

Figure 4 Evidence for the presence of repressor element(s) between −1308 and −338 bp of the GLUT2 promoter in rat hepatocytes cultured in the presence of glucose

Plasmids (10 μg/dish), containing GLUT2 promoter sequences from −1308 to −40 and from −1308 to −338 linked to a minimal thymidine kinase tkCAT promoter, were transfected in hepatocytes by electroporation, as described in Methods section. After an attachment period of 4 h, fresh medium containing 20 mmol/l glucose was added. Cells were harvested 36 h later. The CAT activity was assayed in 100 μg of total cellular protein as described in the Methods section. (**A**) A representative thin-layer chromatograph showing mono- and tri-acetylated and non-acetylated forms of chloramphenicol designated by arrows. PBL-tk CAT, the 5′-region of the minimal tk promoter fused to a CAT reporter gene. (**B**) CAT activity was calculated as the percentage of acetylated form relative to non-metabolized chloramphenicol per 100 μg of cell protein. Results are means ± S.E.M. of six different experiments; $^{*}P < 0.05$ when compared with PBL-tk-CAT.

RESULTS

DNA sequences involved in the stimulation of GLUT2 transcription in response to glucose

We have previously reported that the −338/+49 bp region of the murine GLUT2 promoter contains important sequences involved in the glucose responsiveness of the gene in mhAT3F hepatoma cells [6]. To further characterize the putative glucose-response element(s), we performed transient transfections using 5′-deletions of the −1308/+49 bp region of the GLUT2 promoter linked to a CAT reporter gene in mhAT3F hepatoma cells (Figure 1). In the presence of 17 mmol/l glucose, the highest CAT activity was observed with the −312/+49 bp fragment of the GLUT2 promoter, confirming the presence of a target of

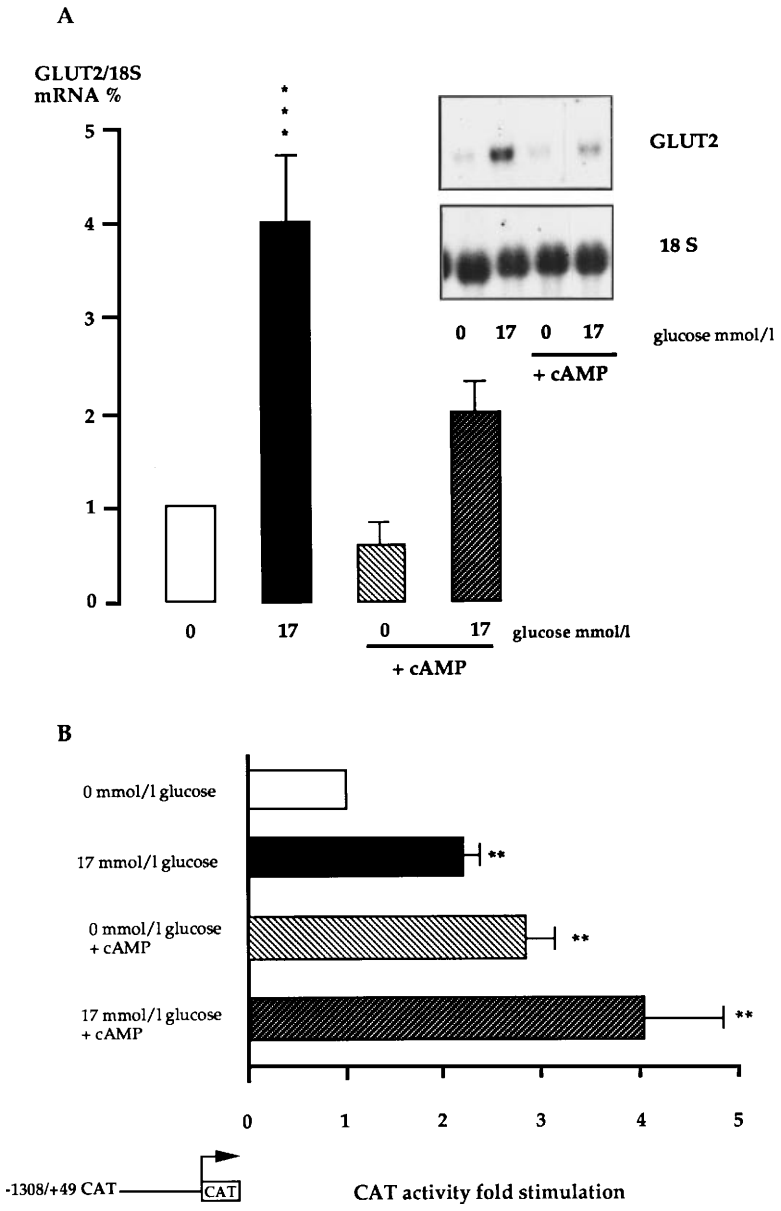

Figure 5 Effect of dibutyryl-cAMP on GLUT2 mRNA concentration and CAT activity of the GLUT2 promoter fragment in the hepatoma cell line mhAT3F

(**A**) Effect of glucose and dibutyryl-cAMP on endogenous GLUT2 mRNA concentration. At 80% confluency, cells were cultured in the absence or presence of 17 mmol/l glucose added or not to 100 μmol/l dibutyryl-cAMP. After 24 h cells were harvested, total RNA extraction and Northern blot analysis was performed as described in the Methods section. Results are expressed as fold increase of GLUT2 mRNA concentration compared with the level observed in cells cultured in the absence of glucose. A representative Northern blot is showed on the right. The GLUT2 mRNA values were normalized by hybridization with an 18 S rRNA probe to correct for RNA loading. Results are means $\pm$ S.E.M. of five different experiments; $^{***}P < 0.001$ when compared with the mRNA level observed in cells cultured in the absence of glucose. (**B**) The quantification of CAT activity driven by the $-1308/+49$ bp fragment of GLUT2 promoter. Cells were transfected with 10 μg of plasmid/dish by lipofection as described in the Methods section. After overnight culture, the medium containing liposomes was replaced by fresh medium with or without 100 μmol/l dibutyryl-cAMP and in the presence or absence of 17 mmol/l glucose. Cells were harvested 24 h later. The CAT activity assayed in 40 μg of cellular proteins was calculated as the percentage of acetylated form relative to non-metabolized chloramphenicol. The effect of glucose or cAMP was then expressed as fold stimulation over the CAT activity measured in cells cultured in the absence of glucose. Results are means $\pm$ S.E.M. of three experiments; $^{**}P < 0.005$ compared with the activity measured in cells cultured in the absence of glucose.

glucose metabolism at between $+49$ and -312 bp. A 2-fold increase in CAT activity is observed with the $-212/+49$ bp fragment of the GLUT2 promoter. CAT activity of the $-212/+49$ bp fragment was not statistically different from $-1308/+49$ bp fragment of the GLUT2 promoter. The activity of CAT was 50% lower than that obtained with the $-312/+49$ bp fragment of the GLUT2 promoter (Figure 1).

Similar experiments were performed in cultured rat hepatocytes. Hepatocytes were transiently transfected with fragments of the GLUT2 promoter, ranging from -1308 to $-212/+49$ bp. In the presence of 20 mmol/l glucose, and as observed in the transformed hepatoma cell line, the highest activity of CAT was obtained with the $-312/+49$ bp fragment (Figure 2).

These data demonstrate that glucose responsiveness of the

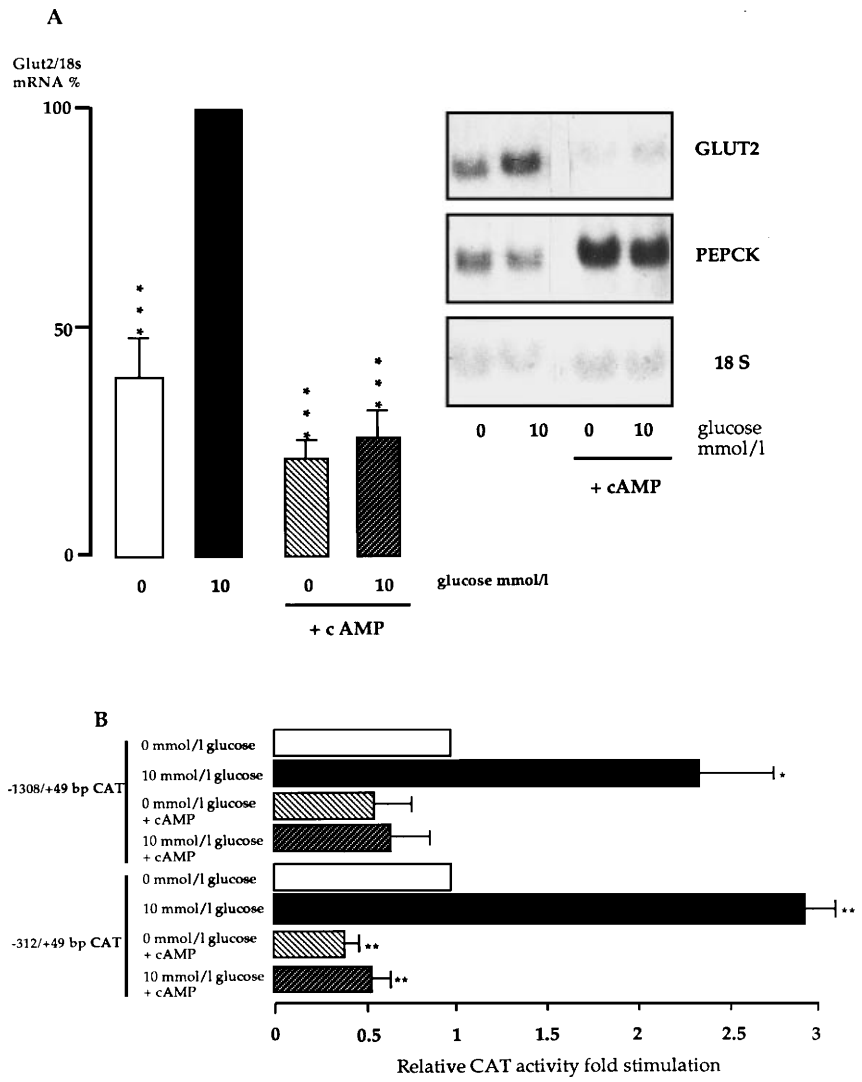

Figure 6 Effect of dibutyryl-cAMP on glucose-induced GLUT2 mRNA concentration and CAT activity of GLUT2 promoter fragments in cultured rat hepatocytes

(**A**) Effect of glucose and dibutyryl-cAMP on endogenous GLUT2 mRNA concentration. Total RNA extraction was performed on the same cell cultures, and Northern blot analysis was performed as described in the Methods section. Results are expressed as fold increase of GLUT2 mRNA concentration compared with the level observed in cells cultured in the absence of glucose. A representative Northern blot is shown on the right. The Northern blot was hybridized with a phosphoenolpyruvate carboxykinase cDNA probe as a control of dibutyryl-cAMP efficiency. The GLUT2 mRNA values were normalized by hybridization with an 18 S rRNA probe to correct for RNA loading. Results are means ± S.E.M. of five different experiments; $^{***}P < 0.001$ when compared with the mRNA level observed in cells cultured in the absence of glucose. (**B**) Quantification of CAT activity driven by the −1308/+49 bp and −312/+49 bp fragments of the GLUT2 promoter. Cells were transfected by electroporation with 10 μg of plasmid/dish, as described in the Methods section. After the attachment period (4 h), fresh medium was added in the presence and absence of 100 μmol/l of dibutyryl-cAMP and 10 mmol/l of glucose. Cells were harvested 24 h later. The CAT activity assayed in 100 μg of cellular proteins was calculated as the percentage of acetylated form relative to non-metabolized chloramphenicol. The effect of glucose or dibutyryl-cAMP was then expressed as fold stimulation over the CAT activity measured in cells cultured in the absence of glucose. Results are means ± S.E.M. of three different experiments; $^{*}P < 0.05$, $^{**}P < 0.005$ compared with the activity measured in cells cultured in the absence of glucose.

murine GLUT2 promoter is partially lost with constructs located 5′ to the −312 bp position, therefore suggesting the presence of repressor element(s) within −1308 to −312 bp of the promoter.

Evidence for a repressor element in the GLUT2 promoter

To test whether repressor element(s) were indeed located in the 5′ region upstream of the GLUT2 promoter, we linked sequences −1308/−40 or −1308/−338 bp of the promoter to a minimal thymidine kinase tkCAT promoter (Figure 3). In the presence of high glucose concentrations, the activity of CAT was 50 % lower when the −1308/−338 bp sequence of the GLUT2 promoter was present in the tkCAT promoter construct, compared with the sequence −1308/−40 bp. These experiments confirmed the presence of a functionally active repressor element(s) upstream of the −338/+49 bp region of the GLUT2 promoter, both in hepatoma cells (Figure 3) and in hepatocytes (Figure 4).

Effect of dibutyryl-cAMP on GLUT2 promoter activity and gene expression

The presence of a consensus CRE element (ACGTCA) located −1069 to −1074 bp upstream of the start site prompted us to test the effect of dibutyryl-cAMP on CAT activity driven by the GLUT2 promoter in mhAT3F cells and hepatocytes (Figures 5 and 6) as well as on endogenous GLUT2 mRNA.

Dibutyryl-cAMP decreased endogenous GLUT2 mRNA concentration ($50 \pm 5\%$) in the absence and in the presence of glucose (17 mmol/l) in mhAT3F cells (Figure 5A). Then we investigated the effects of dibutyryl-cAMP on the $-1308/+49$ bp region of the GLUT2 promoter in hepatoma mhAT3F cells (Figure 5B). Surprisingly, the activity of CAT driven by the $-1308/+49$ bp fragment of the GLUT2 promoter, in the presence of dibutyryl-cAMP, whatever the glucose concentration, remained 2–3-fold higher than the activity measured in the absence of cAMP (Figure 5B). During the time course of the experiments (24 h), dibutyryl-cAMP produced no inhibitory effects on the CAT activity of the construct; meanwhile it decreased the concentration of endogenous GLUT2 mRNA. This effect might be linked to the neoplastic transformation of mhAT3F cells.

Thus, it was important to test whether dibutyryl-cAMP was active in GLUT2 expression in cultured rat hepatocytes. Dibutyryl-cAMP (100 μmol/l), in the presence of theophylline (5 mmol/l), decreased by $66 \pm 4\%$ endogenous GLUT2 mRNA accumulation in hepatocytes cultured in the presence of 10 mmol/l glucose (Figure 6A). Dibutyryl-cAMP inhibited by 34 ± 10 and $74 \pm 8\%$ respectively the CAT activity driven by the $-1308/+49$ bp fragment of the GLUT2 promoter in primary hepatocytes cultured in basal and high-glucose medium (Figure 6B). When hepatocytes were transfected with the $-312/+49$ bp fragment of the GLUT2 promoter (that did not contain the consensus CRE), dibutyryl-cAMP inhibited basal as well as glucose-induced CAT activity by 39 ± 7 and $78 \pm 5\%$ respectively (Figure 6B). Thus, dibutyryl-cAMP inhibited both endogenous GLUT2 mRNA accumulation and CAT activity driven by the $-312/+49$ bp fragment of the GLUT2 promoter in cultured hepatocytes, independently of the presence of the putative CRE.

DISCUSSION

Liver GLUT2 mRNA is increased *in vivo* in rats fed a high-carbohydrate diet [3] or in response to an hyperglycaemic hyperinsulinaemic clamp [2]. The role of hyperglycaemia in the upregulation of liver GLUT2 expression was confirmed *in vitro* in cultured hepatocytes [2,5]. The stimulatory effect of glucose on GLUT2 expression is transcriptionally mediated and requires glucose metabolism [6].

To identify the region of the promoter required for the stimulation of GLUT2 transcription by glucose, transient transfections of hepatoma cells were performed. This cell line was used, since it expresses a significant amount of GLUT2 transporter and since GLUT2 expression is stimulated in the presence of glucose [6]. Moreover, hepatoma cells are more efficiently transfectable than cultured hepatocytes [22]. In the hepatoma cell line mhAT3F, the highest transcriptional activity was carried out by the $-312/+49$ bp fragment of the GLUT2 promoter, and this sequence contained the required *cis*-elements necessary for glucose responsiveness. Lower transcriptional activities were observed with the $-413/+49$ bp and $-1308/+49$ bp fragments of the GLUT2 promoter, suggesting the presence of repressor element(s) upstream of the -338 bp region of GLUT2 promoter. The presence of functionally active repressor element(s) located $-1308/-338$ bp upstream of the start site of the GLUT2 promoter was observed in both mhAT3F cells and hepatocytes, once cloned into a minimal thymidine kinase (tk) promoter driving CAT. This confirmed previous observations peformed in the insulinoma cell line INS-1 [18], suggesting that the repressor element(s) were not tissue-specific, since it was found in cultured hepatocytes, hepatoma cells and insulinoma cells.

Several genes, such as those coding for L-pyruvate kinase (L-PK), S14, acetyl-CoA carboxylase and insulin, have been shown to be transcriptionally regulated by glucose [23–26]. In S14 and L-PK genes, E-boxes (CANNTG, 5 nt, CANNTG) capable of binding a family of transcription factors, USF/MLTF [27], have been reported to be responsible for glucose responsiveness [28–30]. As there was no such consensus sequence in the $-1308/+49$ bp region of the GLUT2 promoter, the glucose responsiveness must be mediated by other unidentified elements. The mechanism of the effect of glucose action on the transcription of the insulin gene involves the presence of a 50 bp sequence, the Far-FLAT mini-enhancer. This sequence confers glucose responsiveness on a non-responsive promoter, after the binding of proteins to multiple sequence elements [31]. Several transcription factors (Pan-1, Lmx-1, IUF-1, IEF-1) have the capacity to bind to this 50 bp sequence [31]. The binding of IUF-1 to the insulin promoter is glucose-dependent, and when glucose metabolism is inhibited by mannoheptulose, an inhibitor of glucokinase, the binding of IUF-1 is abolished [32]. Most of these transcription factors are ubiquitously or tissue-specifically expressed and their abundance is not regulated by diet and hormones. Again, no consensus or degenerated sequence described in the stimulation by glucose of the insulin gene was observed in 1300 bp of the human, murine or rat GLUT2 promoter. In the acetyl-CoA carboxylase gene, the effect of glucose might be mediated by the ubiquitously expressed transcription factors Sp1 or Sp3, which bind to the glucose-response element and transactivate the gene [25]. In the GLUT2 promoter, two Sp1 consensus sites (located at -297 and -256 bp) are present in the $-312/+49$ bp construct, which carries the glucose-response element of the GLUT2 gene. The functional role of Sp1 in the glucose responsiveness of the GLUT2 promoter remains to be established.

The glucose responsiveness could result from the modification of complexes of proteins assembled on DNA-binding motifs. All the genes whose trancription is regulated by glucose might share common factors, but not necessarily common DNA motifs. In the $-1308/+49$ bp region of the GLUT2 promoter, there is no consensus or degenerated sequence, as has been described in other glucose-regulated genes. Thus, the mechanisms involved in the effects of glucose on GLUT2 transcription should be different. Further work is necessary to characterize liver transcription factors capable of binding to the $-312/+49$ bp region of the GLUT2 promoter.

So far, three *cis*-elements have been localized within the -168 bp region of the murine GLUT2 promoter: GTI, II and III [18]. GTI and III bind ubiquitously expressed *trans*-acting factors, which are therefore present in the liver, whereas GTII binds islet-specific nuclear proteins [18]. Therefore, in hepatocytes and mhAT3F cells, the CAT activity observed with the $-212/+49$ bp GLUT2 promoter fragment containing GTI and GTIII is in accordance with the presence of ubiquitously expressed *trans*-acting factors.

The presence of a consensus CRE element -1074 bp upstream from the start site of GLUT2 transcription led us to test the effect of cAMP on GLUT2 expression. In the hepatoma cell line mhAT3F, cAMP increased CAT activity driven by $-1308/+49$ bp fragment of the GLUT2 promoter in the presence or absence of glucose. This was not in agreement with the observed inhibitory effect of cAMP on endogenous GLUT2 expression. In contrast, in cultured hepatocytes, endogenous GLUT2 expression and CAT activity driven by the $-1308/+49$ bp fragment of the GLUT2 promoter were decreased by cAMP. Discrepancies between cultured hepatocytes and the hepatoma cell line mhAT3F in response to cAMP have also been reported for the L-PK gene [22]. Nevertheless, the

reason for such a discrepancy between the primary culture of hepatocytes and the hepatoma cell line mhAT3F could be due to the altered glucose metabolism of transformed cell lines compared with normal cells.

Glucagon and cAMP, as shown here for GLUT2, inhibit the transcription of the L-PK gene *in vivo* and in primary cultures of hepatocytes [23,33]. The inhibitory effect of cAMP on L-PK gene expression requires the E-boxes [29] and is supposed to interact with the binding of transcription factors to this element induced by glucose [34]. Thus in the L-PK gene and in the $-312/+49$ bp region of GLUT2, glucose responsiveness and the capacity to respond to cAMP are located in a single element.

In conclusion, we have demonstrated that the $-312/+49$ bp region of the GLUT2 promoter contains elements required for the stimulatory effect of glucose and for the inhibitory effect of cAMP on liver GLUT2 gene expression in cultured rat hepatocytes. Further experiments are required to determine whether this results from direct inhibition of transcription or from an alteration of glucose metabolism.

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