Cloning and sequencing of the 5′ region of the human glucose-6-phosphatase gene: transcriptional regulation by cAMP, insulin and glucocorticoids in H4IIE hepatoma cells**

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Abstract We have cloned and sequenced the first 1.2 kb of the 5′ region of the human glucose-6-phosphatase gene. Transfection of H4IIE hepatoma cells with the 1.2 kb fragment fused to a luciferase reporter gene demonstrated both basal and hormone responsive luciferase activity. Dexamethasone increased and insulin decreased luciferase activity. Insulin and dibutyryl cyclic AMP both significantly decreased activity in the presence of dexamethasone.

Key words: Glucose-6-phosphatase; Dexamethasone; Insulin; Gene regulation; cAMP

1. Introduction

Glucose-6-phosphatase [EC 3.1.3.9] catalyses the hydrolysis of glucose-6-phosphate to glucose [1,2]. This reaction is the terminal step of both gluconeogenesis and glycogen breakdown in liver. The importance of glucose-6-phosphatase to blood glucose homeostasis is demonstrated by glycogen storage disease type 1a which is a severe metabolic disorder caused by a deficiency of the glucose-6-phosphatase enzyme [3]. Due to its physiological function the enzyme is predominantly expressed in liver and kidney where it is tightly associated with the membrane of the endoplasmic reticulum. The active site of the glucose-6-phosphatase enzyme is inside the lumen of the endoplasmic reticulum and the enzyme is part of a multicomponent system which also consists of transport proteins for the substrate glucose-6-phosphate and the products glucose and phosphate [2,4].

There are three substrate cycles in the liver gluconeogenic and glycolytic pathways and the direction of net flux through these cycles is controlled by the relative activities of phosphoenolpyruvate carboxykinase compared to pyruvate kinase; fructose-1,6-bisphosphatase compared to 6-phosphofructo-1-kinase and glucose-6-phosphatase compared to glucokinase. The gene regulation of five of these enzymes has been extensively studied (phosphoenolpyruvate carboxykinase, pyruvate kinase, fructose-1,6-bisphosphatase, 6-phosphofructo-1-kinase and glucokinase; see [5] for a review). In contrast, much less is known about glucose-6-phosphatase gene, RNA and protein regulation. There are several reasons for the relative lack of knowledge about the regulation of glucose-6-phosphatase. The membrane association and instability in vitro delayed for a long time a molecular characterisation of the glucose-6-phosphatase enzyme protein [4] and the human glucose-6-phosphatase enzyme was cloned only recently [6,7]. Investigation of the function and regulation of the glucose-6-phosphatase enzyme in liver has also been greatly hampered by the lack of cell lines expressing significant levels of glucose-6-phosphatase activity. However, from animal studies it is clear that hepatic glucose-6-phosphatase is regulated. Hepatic glucose-6-phosphatase enzyme activity [1,8], protein levels [8], and mRNA levels [9] all increase greatly in animal models of diabetes mellitus. Administration of glucocorticoids, insulin, thyroid hormone or glucagon to animals has been shown to alter glucose-6-phosphatase activity in microsomes [1,10–13]. In addition, liver glucose-6-phosphatase enzyme activity, protein and mRNA levels are developmentally regulated in mammals [9,14,15]. It therefore seems likely that a number of *cis*-acting elements will be present in the promoter regulatory region of the glucose-6-phosphatase gene to confer these multiple modes of regulation but to date the 5′ region of the gene has not been characterised and very little is known about the mechanisms of hormonal regulation of glucose-6-phosphatase gene expression [16].

In this study we report the cloning and sequencing of the 5′ region of the human glucose-6-phosphatase gene. The expression in H4IIE hepatoma cells of a construct between the first 1.2 kb of this region and a luciferase reporter gene was regulated by dexamethasone, insulin and dibutyryl cyclic AMP (dBcAMP).

2. Materials and methods

2.1. Materials

Restriction endonucleases, modifying enzymes, plasmid DNA purification kit (Wizard), and random prime labelling kit (Prime a Gene system) were from Promega. All radioisotopes for labelling probes ([α-^{32}P]dCTP) and sequencing ([α-^{35}S]dATP) and Hybond N$^+$ were purchased from Amersham. Insulin, dibutyryl cAMP, dexamethasone were purchased from Sigma. Kodak Biomax MR Scientific Imaging Film was used for all autoradiography.

2.2. Library screening

Duplicate high density filters containing DNA from a human chromosome 17 specific genomic cosmid library (Reference Library, ICRF) were probed with a full length cDNA of the human glucose-6-phosphatase catalytic subunit [6]. Four positives were identified, two of which (c105H1083 and c105H03159) contained overlapping inserts of the glucose-6-phosphatase gene. A combination of restriction mapping and Southern blotting was then used to identify overlapping fragments of the 5′ region of the gene. Appropriate fragments were gel isolated and subcloned into the polylinker region of the plasmid pGem7(+) (Promega). Both strands of the recombinant plasmids were

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**Sequence data from this article have been deposited with the EMBL database under Accession Number (still to be obtained from EMBL).

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; HNF, hepatocyte nuclear factor; CRE, cAMP response element; IRE, insulin response element; GRE, glucocorticoid response element.

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sequenced by the dideoxy chain termination method [17] using the Sequenase Version 2.0 sequencing kit (USB) utilising the T7 and SP6 promoter primers. When necessary deletion mutants of the recombinants were generated using exonuclease III (Erase-a-Base system, Promega).

2.3. Construction of the reporter gene plasmids

A 0.6 kb *Bam*HI fragment (−498 to +114) of the promoter region was cloned into the *Bam*HI site of the pT7 Blue vector (Novagen). The 3′ 57 bases (which code for the first 19 amino acids of the glucose-6-phosphatase protein) were deleted from this construct using exonuclease III. The remaining insert was cloned into the *Eco*RI/*Hin*dIII site of pGem7 and a 0.75 kb *Sac*I/*Bam*HI fragment of the c105H03159 cosmid was cloned upstream of this. This 1.25 kb insert was then cloned into the *Sac*I/*Xho*I site of the pGL3 Basic vector (Promega). The reporter gene construct (pGL3-1.3) was verified by restriction mapping and sequencing.

2.4. Cell culture and transient expression assays

H4IIE cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal calf serum. One day before the transfection 600 000 cells were plated out per culture dish (35 mm). 4 h before the transfection the cells were fed with fresh culture medium. Transfection was performed with the $CaPO_4$/DNA co-precipitation method according to Docherty and Clard [18], with the modification that per dish 250 μl of the precipitate was used containing 10 μg DNA of the reporter gene and 1.5 μg pSV-β-galactosidase control vector (Promega) as internal standard for the transcription efficiency. After 18 h the DNA-containing medium was replaced by DMEM/15% glycerol for 2 min [19]. Then the cells were washed and cultivated for 4 h in DMEM. Subsequently the medium was replaced by DMEM containing dexamethasone, insulin or dibutyryl cAMP. 46 h after the transfection the cells were washed twice with phosphate-buffered saline and lysed with reporter lysis buffer. Luciferase activity was determined with a Bio-Orbit 1250 Luminometer (LKB) using the Luciferase Assay Reagent (Promega). β-Galactosidase activity was determined at 420 nm with *o*-nitrophenyl β-D-galactopyranoside as substrate [20]. Statistical analysis was performed using the InStat program (version 2.01) for Macintosh computers.

3. Results and discussion

The first 1.2 kb of the 5′ region of the human glucose-6-phosphatase gene was isolated and sequenced in both directions. The sequence obtained is shown in Fig. 1. The 5′ promoter region of the glucose-6-phosphatase gene contained a perfect consensus sequence for a TATA box. There are also a number of sequences with homologies to putative binding sites for liver enriched transcription factors within the 5′ flanking region (Fig. 1). These include three C/EBP sites, an HNF 1, an HNF 3, an HNF 4 and five HNF 5 sites together with three binding sites for the general transcription AP-1 and two CRE sites and two IRE sites. In addition a weak putative GRE binding site was found at −332. While many of the putative binding sites in the 5′ promoter region of the glucose-6-phosphatase gene may not of course be functional in vivo, the presence of so many sites could indicate that this might be a highly regulated portion of the 5′ region of the gene.

To determine if the sequenced region contained a functional promoter we constructed a reporter gene plasmid (pGL3-1.3) containing this portion of the glucose-6-phosphatase 5′ region (−1227 to +57) and transfected it into H4IIE hepatoma cells. Transfection of plasmid pGL3-1.3 into H4IIE cells resulted in three-fold greater luciferase activity than cells transfected with basic vector (pGL3) alone (Table 1). It also demonstrated that this 5′ region of the glucose-6-phosphatase gene does contain hormone response elements as it was regulated by the synthetic glucocorticoid dexamethasone, the cAMP mimetic dibutyryl cAMP and insulin (Table 1). Dexamethasone increased the luciferase activity in pGL3-1.3 transfected cells 10-fold, insulin significantly decreased it to levels which were very similar to the basal expression of luciferase activity of the

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                                        AP 2
-1227 GAGCTCAGGA   ATTCAAGACC   AGCCTGGGCA   ACATGGAAAA
                                             AP 2
-1187 ACCCCATCTC   TACAAAAGAT   AGAAAAATTA   GCCAGGCATG
-1147 GTGGCGTGTG   CCTGTGGTCC   CAGCTACTCA   GGAGGCTGAG
                                             AP 1
-1107 GTGGGAGGAT   CACATTAGCC   CAGGAGGTTG   AGGCTGCAGT
                                             AP 1
-1067 GAGCCGTGAT   TATGCCACTG   CACTCCAGCC   TGGGAGACAG
-1027 AGTGAGACCC   TGTTTCAAAA   AAAAGAGAGA   GAAAATTTAA
                HNF 5
- 987 AAAAGAAAAC   AACACCAAGG   GCTGTAACTT   TAAGGTCATT
                C/EBP
- 947 AAATGAATTA   ATCACTGCAT   TCAAAAACGA   TTACTTCTGG
- 907 CCCTAAGAGA   CATGAGGCCA   ATACCAGGAA   GGGGGTTGAT
                             AP 2
- 867 CTCCCAAACC   AGAGGCAGAC   CCTAGACTCT   AATACAGTTA
- 827 AGGAAAGACC   AGCAAGATGA   TAGTCCCCAA   TACAATAGAA
- 787 GTTACTATAT   TTTATTTGTT   GTTTTTCTTT   TGTTTTGTTT
                             HNF 3
- 747 TGTTTTGTTT   TGTTTTGTTT   TAGAGACTGG   GGTCTTGCTC
- 707 GATTGCCCAG   GCTGTAGTGC   AGCGGTGGGA   CAATAGCTCA
                                  C/EBP        IRE
- 667 CTGCAGACTC   CAACTCCTGG   GCTCAAGCAA   TCCTCCTGCC
- 627 TCAGCCTCCT   GAATAGCTGG   GACTACAAGG   GTACACCATC
        HNF 5
- 587 ACACACACCA   AAACAATTTT   TTAAATTTTT   GTGTAGAAAC
                                  AP 2
- 547 GAGGGTCTTG   CTTTGTTGCC   CAGGCTGGTC   TCCAACTCCT
                             IRE
- 507 GGCTTCAAGG   GATCCTCCCA   CCTCAGCCTC   CCAAATTGCT
                   AP 1           HNF 5
- 467 GGGATTACAG   GTGTGAGCCA   CCACAACCAG   CCAGAACTTT
        C/EBP
- 427 ACTAATTTTA   AAATTAAGAA   CTTAAAACTT   GAATAGCTAG
                                  HNF 5
- 387 AGCACCAAGA   TTTTTCTTTG   TCCCCAAATA   AGTGCAGTTG
                        CRE           HNF 4
- 347 CAGGCATAGA   AAATCTGACA   TCTTTGCAAG   AATCATCGTG
                GRE
- 307 GATGTAGACT   CTGTCCTGTG   TCTCTGGCCT   GGTTTCGGGG
                             AP 2
- 267 ACCAGGAGGG   CAGACCCTTG   CACTGCCAAG   AAGCATGCCA
              HNF 1
- 227 AAGTTAATCA   TTGGCCCTGC   TGAGTACATG   GCCGATCAGG

                                             C/EBP
- 187 CTGTTTTTGT   GTGCCTGTTT   TTCTATTTTA   CGTAAATCAC
- 147 CCTGAACATG   TTTGCATCAA   CCTACTGGTG   ATGCACCTTT
                             HNF 5
- 107 GATCAATACA   TTTTAGACAA   ACGTGGTTTT   TGAGTCCAAA
                             CRE
-  67 GATCAGGGCT   GGGTTGACCT   GAATACTGGA   TACAGGGCAT
      TATA-BOX
-  27 ATAAAACAGG   GGCAAGGCAC   AGACTCATAG   CAGAGCAATC
                                             CRE
   14 ACCACCAAGC   CTGGAATAAC   TGCAAGGGCT   CTGCTGACAT
   54 CTTCCTGAGG   TGCCAAGGAA   ATGAGGATGG   AGGAAGGAAT
   94 GAATGTTCTC   CATGACTTTG   G114
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Fig. 1. The nucleotide sequence of the proximal promoter region of the human glucose-6-phosphatase gene (−1227 to +114). Various putative response element motifs are indicated above the nucleotide sequence. The transcription start site is at +1 and the translational start site at +80 [6]. The transcription start site is marked with an X and the TATA box is underlined. CRE = cAMP response element; C/EBP = CCAAT/enhancer binding protein; GRE = glucocorticoid response element; HNF = hepatocyte nuclear factor; IR-E = insulin response element. The reporter gene construct pGL3-1.3 contains a fragment containing nucleotides −1227 to +57 of this sequence.

Table 1
Effect of dexamethasone, insulin and dibutyryl cAMP on the transcriptional activity of the 1.2 kb glucose-6-phosphatase promoter

Vector	Mediator	Relative luciferase activity (fold enhancement over pGL3 basic vector)
pGL3 basic vector	none	1
pGL3-1.3	none	3.2 ± 0.6
pGL3-1.3	dibutyryl cAMP	3.6 ± 0.8
pGL3-1.3	dexamethasone	38.7 ± 7.9**
pGL3-1.3	insulin	1.3 ± 0.4*
pGL3-1.3	dexamethasone+dibutyryl cAMP	8.3 ± 0.8**^
pGL3-1.3	dexamethasone+insulin	2.2 ± 0.4^+
pGL3-1.3	dibutyryl cAMP+insulin	2.4 ± 0.3+

Luciferase activity was assayed 46 h after transfection of H4IIE cells with the pGL3-1.3 construct. 24 h before harvesting the cells were maintained under hormonal and intracellular mediator influence as described in the Table. The following concentrations were used: insulin, 500 nM; dexamethasone, 1 μM; dibutyryl cAMP, 500 μM. Luciferase activities were corrected for β-galactosidase activities. The results are expressed as fold stimulation compared to the luciferase activity measured after transfection with pGL3 basic vector, which was set as 1.0. All values represent the mean (S.D.) of three independent experiments, which were all carried out in triplicate.
*Significantly different from pGL3-1.3 alone $*P < 0.01$, $**P < 0.005$; ^significantly different from pGL3-1.3 plus dexamethasone $^P < 0.005$; +significantly different from pGL3-1.3 plus insulin $^+P < 0.05$.

pGL3 basic vector (Table 1). Insulin significantly decreased the stimulation of luciferase activity by dexamethasone. In contrast, dibutyryl cAMP alone did not significantly alter the luciferase activity in the pGL3-1.3 transfected H4IIE cells (Table 1) and it only had a significant effect when it was used in combination with either dexamethasone or insulin. Dibutyryl cAMP and dexamethasone have previously been shown to synergistically increase the expression of another key gluconeogenic enzyme (phosphoenolpyruvate carboxykinase) [5]. It was therefore surprising that dibutyryl cAMP decreased both the stimulation of luciferase activity by dexamethasone and the inhibition of luciferase activity by insulin (Table 1). The regulation of the expression of the reporter gene construct (Table 1) is consistent with the recent report, based on Northern blots of FAO cell RNA, that dexamethasone increases glucose-6-phosphatase mRNA levels and insulin decreases them [21]. There have been some reports in the literature indicating that glucocorticoids (dexamethasone) increase liver glucose-6-phosphatase enzyme activity and others showing that it does not (e.g. [1,12,14,22–27]). A simple explanation for these apparently conflicting findings would be that the effect of dexamethasone also depends on the levels of other hormones like insulin and glucagon as illustrated in Table 1.

In liver glucokinase catalyses the formation of glucose-6-phosphate which is the opposite reaction to that of glucose-6 phosphatase. The molecular mechanisms in the long-term control of the glucose/glucose-6-phosphate substrate cycle during the switch between glycolysis and gluconeogenesis are unknown [16]. The transcription of the glucokinase gene is stimulated by insulin and inhibited by glucagon [5,16]. The data presented here suggest that during the switch between glycolysis and gluconeogenesis insulin might act at the transcriptional level as the predominant regulator in the control of the glucose/glucose-6-phosphate substrate cycle.

Recently approximately 20 different mutations have been found in the coding region of the human glucose-6-phosphatase gene in patients with glycogen storage disease type 1a, e.g. [7]. However in about 17% of cases of glycogen storage disease type 1a no mutations were found in the coding region of the glucose-6-phosphatase gene [7]. A possible explanation is that some patients with glycogen storage disease type 1a have mutations in the promoter region of the gene. The sequence data in Fig. 1 will facilitate future studies to determine if some patients with glycogen storage disease type 1a have mutations in the 5′ region of the glucose-6-phosphatase gene.

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